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ABSTRACTS

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Abstracts for the oral presentations

Mechanism of muscle contraction

Direct evidence for the cross-bridge lever arm mechanism in muscle contraction studied using the gas environmental chamber and site-directed antibodies

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During muscle contraction, the cross-bridges in myosin filaments first attach to actin filaments in the form of MADPPi, undergo a conformational change (power stroke) associated with release of Pi and ADP to produce sliding between actin and myosin filaments, and then detach from actin filaments upon binding with next ATP. The detached cross-bridges undergo a reversed conformational change (recovery stroke) associated with reaction, MATP → MADPPi. It is suggest that the cross-bridge strokes result from rotation of the cross-bridge lever arm domain around the converter domain, while the catalytic domain remains rigid. To ascertain the validity of the lever arm mechanism in muscle contraction, we recorded ATP-induced movement at different regions within individual cross-bridges in living myosin filaments, using the gas environmental chamber, with which biological macromolecules can be kept in living state in an electron microscope. Three different regions of the cross-bridge were position-marked with site-directed antibodies; antibody 1 to the distal catalytic region, antibody 2 to the interface between the catalytic and converter domains, and antibody 3 to the boundary between the lever arm domain and myosin subfragment 2. We have found that the average amplitude of ATP-induced movement was 6.14 nm at both the distal catalytic domain and the catalytic-converter domain interface, and 3.77 nm at the lever arm domain-subfragment 2 boundary, providing the first direct evidence for the cross-bridge lever arm mechanism.

Muscle force generation examined by laser temperature-jump and ramp shortening

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In maximally active muscle, the tension decline during a ramp shortening shows an initial inflection (change in slope) representing the force generation in the (pre-stroke) crossbridges when negative strained (Ford et al. J Physiol 269:441, 1977; Roots et al. JMRCM, 28:123, 2007). We examined the effect of a rapid temperature jump (<0.2 ms, 3–4°C T-jump) in maximally Ca-activated rabbit psoas muscle fibres at 8–9°C when applied coincident with the onset of ramp-shortening at different velocities (V, range ~0.01L0/s to ~1.5L0/s). Without ramp shortening (isometric state), a T-jump induced a biphasic tension rise consisting of a fast (~50/s, labelled phase 2b) and a slow (~10/s, phase 3) component. With ramp shortening, the T-jump force generation was extracted by subtracting the tension response to a ramp shortening without a T-jump at the same velocity. Such "difference tension records" clearly showed a bi-component tension rise (fast 2b and slow 3) to a T-jump which, in comparison to isometric, were temporally separate at the higher velocities. The rates of both components increased linearly with increase of velocity so that phase 2b rate (force generation) near Vmax (~1.25L0/s) reached >600/s, about 10-times faster than in isometric. Our results suggest that the power-stroke is endothermic and that, when exposed to negative strain (at the onset of ramp shortening), the T-jump tension generation can be almost as fast as quick tension recovery in the step length-release experiments.

Qualitatively distinctive myosin head attachment mechanisms at high and low levels of activation in fast twitch muscle fibres

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Force-generating attachments of myosin heads were investigated by applying small perturbations of myosin head pulling cycles in stepwise stretch experiments on fast-twitch single skeletal muscle from rat (Fisher 344) and frog (*Xenopus laevis*). The results show a

qualitatively distinct mechanism of cross-bridge attachment at low levels (<30% maximal activation) and at high levels (>80% maximal activation) of activation. The mechanism of cross-bridge attachment at high level of activation is up to 30-fold faster than that occurring at low levels of activation and the two mechanisms co-exist at intermediate levels of activation (30–80% maximal activation). The sudden occurrence of the fast mechanism of cross-bridge attachment when the level of activation is increased above about 30% maximal activation can be explained by a facilitation process whereby one attached myosin head substantially accelerates the attachment of a second myosin head within its neighborhood. The much faster cross-bridge attachment mechanism at high levels of activation does not occur in slow muscle fibres suggesting that the facilitation process operates only in fast-twitch fibres.

The absence of cardiac myosin binding protein-C prolongs myosin cross-bridge duration and enhances cross-bridge sensitivity to inorganic phosphate

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A high percentage of familial cardiomyopathies originate in mutations of cardiac myosin binding protein-C (cMyBP-C), a thick filament-associated protein whose absence has been shown previously to enhance contractile performance and reduce relaxation. It has been proposed that cMyBP-C normally provides an elastic and viscous load that slows contractile performance yet provides a means for storing elastic recoil energy which facilitates sarcomere relengthening. We investigated the removal of cMyBP-C on myosin cross-bridge duration, ton, using length perturbation analysis of chemically-skinned myocardial strips from mice, comparing results from an effective null for cMyBP-C (t/t) with that of non-transgenic (NTG) and transgenic wild-type (WTt/t) controls, the latter with cMyBP-C expressed into t/t. Mice were PTU-treated to produce uniform populations containing 10/90 ratio of α/β-myosin heavy chain. We found that ton was significantly longer in the t/tPTU (6.87 ± 0.85 ms) compared to NTGPTU (4.28 ± 0.45 ms) examined at 37°C, 5 mM ATP and 0.25 mM inorganic phosphate (Pi). Raising Pi to 4 mM significantly shortened ton in both the t/tPTU (5.30 ± 0.68 ms) and NTGPTU (3.71 ± 0.41 ms), although the effect of Pi was significantly more pronounced in the t/tPTU. Results for ton and its sensitivity to Pi were similar among the NTG and WTt/t not treated with PTU. These results suggest that the relatively prolonged ton and enhanced sensitivity to Pi in the absence of cMyBP-C is due to a prolonged ADP state in the cross-bridge cycle independent of myosin isoform. Thus t/t-related changes in strain-sensitive Pi and/or ADP binding affinity due to altered thick filament stiffness, manifest as increased ton, could account for the enhanced contractile performance and reduced relaxation reported previously in whole hearts from t/t mice. Funded by NIH grant HL59408.

Modulation of contractile activity by MLC2 O-GlcNAcation

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Recent experiments suggest that glycosylation by O-GlcNAc might be as much important as phosphorylation in the regulation of contractile activity. Thus, O-GlcNAc can modulate the calcium activation parameters since a decrease in calcium activation parameters is observed when O-GlcNAc-depending protein–protein interaction are abolished, while an increase of the calcium affinity is measured after a pharmacological increase of O-GlcNAc level. In order to analyze how hyperglycosylation could modulate the calcium activation parameters, we studied the variation of O-GlcNAcation on proteins isolated from soleus skinned fibers. Among proteins previously described as O-GlcNAc (i.e. myosin heavy and light chains, actin, and tropomyosin), we have also identified some structural proteins (actinin alpha 2 and desmin) as well as some proteins of the troponin complex (slow and fast isoforms of TnI and TnT) to be O-GlcNAcylated. We measured an increase of O-GlcNAcation on TnTf and MLC1f, and slow and fast isoforms of MLC2 in hyperglycosylated biopsies. This increase of O-GlcNAcation is associated to the modification of phosphorylated MLC2 pattern, suggesting an interplay between the two post-translational modifications. Since it is difficult to attribute to fast isoforms the effect observed in slow fibers, we postulate that the increase in calcium affinity observed in hyperglycosylated fibers might involved the increase in O-GlcNAcation of the slow MLC2. The potential sites of O-GlcNAcation on the slow isoform of MLC2 (Ser1 and Thr146) are different from the one identified as phosphorylated (Ser14), contributing to the hypothesis of the existence of a dynamic cross talk between phosphorylation and glycosylation which could modulate MLC2 function.

Experimental evidence for a two-step Ca^{2+}-induced conformational change of skeletal muscle troponin in rabbit psoas myofibrils

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To investigate the kinetics of Ca^{2+}-controlled conformational changes of skeletal Troponin I (TnI) inside the sarcomere, TnI was purified from rabbit skeletal muscle and labelled with the fluorescent marker IAF at Cys134 on its helix-4, which is known to interact with actin-tropomyosin to inhibit acto-S1 ATPase at low [Ca^{2+}]. The labelled TnI was then incorporated together with the other two skeletal troponin subunits (TnC and TnT) as a complex (TnIAF) into rabbit psoas myofibrils. Rapid mixing of these myofibrils with Ca^{2+} in a stopped-flow apparatus revealed a biphasic fluorescence transient with a rapid decrease (observed rate constant kobsfast = 900 s^{-1}) and a slower increase (kobsslow = 110 s^{-1}). Rapid reduction of [Ca^{2+}] induced a monophasic decay with a rate constant koff = 16 s^{-1}. Kinetics are consistent with a two step conformational change of the helix-4 of TnI in which a fast change is closely associated with the rapid binding of Ca^{2+} to TnC and a slower one is likely attributed to the Ca^{2+}-induced dissociation of helix-4 from actin and therefore the latter might report the regulatory switch of Tn. The different polarity of the two phases allowed for the first time to resolve the kinetics of the changes associated with Ca^{2+}-binding. Our data also suggests that while binding Ca^{2+}, Tn is in a rapid equilibrium between switched-on and switched-off states.

Role of tropomyosin (Tm) isoforms in skeletal muscle thin filament regulation

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We investigated the influence of recombinantly expressed $\alpha\alpha$-Tm vs. $\beta\beta$-Tm dimers on myofibril contraction and thin filament sliding in

vitro motility assays. We previously described methods to remove endogenous Tm and troponin (Tn) from myofibrils and replacement with exogenous regulatory proteins (Scellini et al. Biophys J 96(3): 228a, 2009). Replacement, evaluated by SDS-PAGE, was 92 ± 2% for αα-Tm and 77 ± 2% for ββ-Tm reconstitutions. Maximal isometric tension (P0), Ca^{2+} sensitivity of tension (pCa50) and cooperativity of activation (nH) did not differ between the two groups, but rates of activation (*k*ACT) and tension redevelopment (*k*TR) were reduced by ~30%. Maximal sliding speed (pCa 5) and fraction of moving thin filaments on rabbit skeletal heavy meromyosin surfaces was not affected by Tm composition, but pCa50 of speed was decreased with ββ-Tm and regulation required ~2-fold more αα-Tm vs. ββ-Tm, suggesting impaired regulation. This idea was supported in myofibrils where ββ-Tm reconstitution compromised regulation at pCa 9.0. Slack sarcomere length (s.l.) was significantly shorter (1.83 ± 0.04 μm) compared to αα-Tm (2.28 ± 0.02 μm). and the passive tension-s.l. relationship was left-shifted with ββ-Tm vs. αα-Tm. Both of these mechanical features were greatly reduced by 10 mM BDM, suggesting high contents of ββ-Tm may result in Ca^{2+} independent activation of reconstituted thin filaments. Telethon-Italy GGP07133, EU STREP P. BIG-HEART 241577 (CP), HL065497 (MR).

Cytoskeleton & trafficking

The sarcomeric M-band in strain sensing and autophagic protein turnover: implications for muscle diseases

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In the sarcomeric M-band, the giant ruler proteins titin/connectin and obscurin, its small homologue obscurin-like-1 (obsl1), and the myosin cross-linking protein myomesin form a ternary complex contributing to its mechanical functions. M-band titin also contains a protein kinase domain (TK) with an unusual autoinhibited structure that has been implicated in mechanosignalling to ligands involved in transcriptional regulation and autophagic protein turnover. Autophagy is a key process in muscle for removing toxic protein aggregates and defective organelles, but also for the recycling and replacement of contractile sarcomeres and supporting membrane structures during muscle development and adaptation. Hereditary and acquired muscle diseases with muscle atrophy lead to protein aggregates, of which the autophagy-linked p62/A170/SQSTM1 protein is a key component. We have identified a novel component, the homologous but distinct protein nbr1, to be also involved in autophagy. Nbr1 and p62 bind to the myofibrillar TK domain. This link is implicated in the control of muscle mass, structure and metabolism by regulating autophagy during muscle loss and growth in response to work load. Mutations in the titin immunoglobulin domain M10, which interacts with the N-terminal Ig domains of obscurin and obsl1, and in the kinase domain itself lead to hereditary skeletal and cardiac muscle diseases with perturbed protein turnover. Disruption of M-band mechanics thus seems to impinge on mechanosignalling and to disrupt ordered protein turnover of the sarcomere.

The function of obscurin in the assembly of the sarcomere in *Drosophila* flight muscle

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Drosophila obscurin is an M-line protein distributed throughout the diameter of the sarcomere. The protein has 21 Ig domains and two kinase domains near the C-terminus. We have studied the effect of reducing expression of obscurin on the symmetry of the filament lattice in flight muscle (IFM). Insertion of a P-element in the first intron of the gene resulted in reduced expression of obscurin and homozygous mutants were unable to fly. The M-line was missing and the H-zone shifted relative to the centre of the sarcomere. Thick filaments were asymmetrical; the polarity of thin filaments followed that of the neighbouring thick filament, resulting in abnormally long or short thin filaments. Obscurin RNAi expressed in IFM produced a phenotype with greater asymmetry in thick filaments. Electron microscopy of isolated filaments showed a bare zone that was shifted away from the centre of the filament, confirming that abnormal sarcomere structure is due to incorrect thick filament assembly. Binding partners of kinase domains in obscurin have been identified. Kinase 1 binds ball, another kinase, and kinase 2 binds MASK, containing ankyrin repeats. RNAi lines of both ball and MASK resulted in IFM sarcomeres with shifted H-zones, similar to those in obscurin mutants. In MASK mutants, Z-discs were also affected. Thus, correct sarcomere assembly depends on an obscurin anchor for thick filaments in the centre of the sarcomere, and on associated ligands likely to have a signalling function.

Obscure links and cullin(g) ends

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Obscurin, a giant protein of the obscurin family, participates in a variety of signalling and scaffolding functions in cross-striated muscles. Recently, obscurin and its close homologue Obsl1 were shown to interact with titin and myomesin at the sarcomeric M-band. Pathological titin mutations that cause limb-girdle muscular dystrophy (LGMD2J) display decreased binding to obscurin/Obsl1. To investigate obscurin functions, and identify pathways that may be involved in LGMD2J progression, we generated the obscurin knockout mouse. Lack of obscurin leads to changes in SR architecture and disruption of small ankyrin- 1.5 (sAnk1.5) expression and localisation. While searching for further sAnk1.5 binding partners, we identified a novel substrate-adaptor that associates sAnk1.5 to cullin-mediated protein turnover, indicating that reduced expression levels of sAnk1.5 in obscurin knockout muscles are due to nedd8- and ubiquitin-dependent protein degradation. Intriguingly, cullin E3-ligases, their substrate adaptors and modulators are also altered in obscurin knockouts. Deciphering the complex links of obscurin proteins and their binding partners with protein degradation mechanisms may provide insights into molecular pathways associated with LGMD2J progression.

In vivo imaging of protein dynamics in damaged skeletal muscle cells in zebrafish reveals an annexin based wound repair mechanism

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Skeletal muscle cells are constantly facing damaging conditions, arising primarily from eccentric contraction. In these conditions the cells acquire small plasma membrane lesions, which are then actively repaired. Due to the spatiotemporally very limited nature of the event, the process has not

been visualized and studied at the whole organism level so far. We used muscle specific unc45b promoter to drive the expression of various fluorescently labeled genes in transparent zebrafish larvae. By causing precise membrane lesions with two-photon laser, we have recorded intracellular protein movements in damage conditions. We demonstrate that annexin proteins are strictly cytoplasmic, yet relocate to the damage site, where they form a tight network, potentially acting as a sieve to capture vesicles in the area. In contrast to previous cell culture models, we did not detect strong lysosome accumulation to the lesion. Various endo- and exocytotic vesicles could be detected near the lesion, yet not at high level. Rab27a has been shown to be up regulated in dysferlin deficient muscles. The zebrafish rab27a has very limited expression in the muscle tissue. Curiously, reduction in Rab27a level leads to mild myopathy and overexpressed Rab27a accumulates to the wound site. Finally, we provide evidence that annexin exposure at the lesion could act as intercellular signaling mediator, by attracting glycoprotein tenascin C matrix.

Myosin Va cooperates with PKA RIα to mediate nerve-dependent maintenance of the mouse endplate in vivo

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Myosin V motor proteins facilitate recycling of synaptic receptors, including AMPA and acetylcholine receptors, in central and peripheral synapses, respectively. To shed light on the regulation of receptor recycling we employed in vivo imaging of mouse neuromuscular synapses. We found that myosin Va cooperates with PKA on the postsynaptic side to maintain size and integrity of the synapse as well as the lifetime of synaptic acetylcholine receptors (AChRs) in a nerve activity-dependent manner. Myosin Va and PKA colocalized in punctate, subsynaptic enrichments. These accumulations were crucial for synaptic integrity and proper cAMP signaling, and were dependent on AKAP function, myosin Va and an intact actin cytoskeleton. In dystrophic muscles lacking the actin-organizing protein dystrophin, localization of myosin Va and PKA, stability of AChRs, integrity of synapses and cAMP signaling were subverted. Our data imply a model where neuronal ligands trigger local activation of PKA, which in turn controls proper turnover of AChRs. Thereto, myosin Va mediates correct positioning of PKA in a postsynaptic microdomain, presumably by anchoring PKA to the subsynaptic actin cytoskeleton.

A proteomic approach for identifying novel interaction partners of nebulin's SH3 domain

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Nebulin is a giant highly modular protein, which stretches along the thin filament in skeletal muscle and plays important roles in regulation of thin filament length, force generation, and maintenance of sarcomeric integrity during skeletal muscle contraction. Furthermore, an SH3 domain in nebulin's extreme C-terminal end has been suggested to be involved in signaling at the Z-line. Mutations in nebulin are the major cause of nemaline myopathy, a non-dystrophic neuromuscular disorder characterized by muscle weakness and the presence of "nemaline" rod bodies in the fibers. Interestingly, patients have been described with a premature stop codon, resulting in ablation of nebulin's C-terminal SH3 domain, suggesting its importance for normal muscle function. To identify novel binding partners of nebulin's SH3 domain, we performed a pull-down assay. Briefly, his-tagged nebulin SH3 domain was incubated with protein lysate from differentiated C2C12 cells and immobilized on a Ni^{2+} column. Eluted proteins were subsequently identified by separation by SDS-PAGE, followed by trypsin digestion and ESI tandem mass spectrometry. Several of the identified potential interaction partners contained typical proline-rich SH3 binding motifs as determined by the bioinformatics tool "SH3 hunter", including AHNAK/desmoyokin, Ankrd2, MLP, and NACAM. In particular, nebulin's interaction with AHNAK, known to be associated with the L-type Ca^{2+} channel and S100B, suggests its role in calcium homeostasis.

Ion channels

Ca^{2+} influx under voltage control in skeletal muscle: still open questions

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Ca^{2+} homeostasis is under the tight control of membrane potential in skeletal muscle. In active muscle fibers, the massive release of Ca^{2+} from the sarcoplasmic reticulum as well as transmembrane Ca^{2+} influx is triggered and controlled by the depolarization of the sarcolemma. In resting muscle, a Ca^{2+} influx is also known to occur through the sarcolemma and the transmembrane voltage sets the electrochemical gradient that drives Ca^{2+} in. For several years, our group has been studying transmembrane Ca^{2+} influx in resting and active single mouse muscle fiber, systematically under voltage control. Using the cell-attached configuration of patch clamp in single mouse muscle fibers, we have demonstrated that local hyperpolarization potentiates Ca^{2+} influx and induces subsarcolemmal Ca^{2+} load revealed by transient opening of Ca^{2+}-activated K^+ channels. By combining the Mn^{2+} quenching method and silicone voltage clamp, we also showed that resting divalent cations influx depends on external cations concentrations and membrane potential, but was too small to be resolved as a macroscopic current. In active muscle fibers, trains of action potentials considerably increased divalent cations entry through an electrically silent pathway independent of L-type channels which provided one quarter of the global influx at +30 mV. Within this context, the nature and the physiological role of the Ca^{2+} influx pathways involved during muscle excitation and at rest still remain open questions.

Upon channels responsible for the electrical bistability of the fiber membrane

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Membrane bistability means that the resting potential P of normal fibers can jump between two stable values, P1 and P2. While fibers in the P1 state follow the predictions of the Goldman-Hodgkin-Katzequation, P2 is about −60 mV and its value but not its frequency is largely independent of $[K^+]o$. Fibers in the P1 state can generate

action potentials. Fibers in the P2 state are paralyzed because most voltage-gated Na^+ channels are inactivated. In normal muscle at 4 mM $[K^+]o$, 10% of the fibers are in the P2 state, but its frequency progressively increases with lower $[K^+]o$. At rest, hyperpolarizing currents outbalance depolarizing currents. A reduction of hyperpolarizing currents or an increase in depolarizing currents enhances the relative frequency of the P2 state. This state becomes transiently or permanently predominant if the (i) hyperpolarizing Cl^- current is reduced (ii) Nav1.4 inward sodium current through the central (alpha) channel pore is increased in a certain voltage range (iii) depolarizing Na^+ inward current through the omega pore is increased (iv) hyperpolarizing K^+ current is reduced. In corresponding channelopathies, the $[K^+]o$ reduction required to cause the P2 state is smaller than in normal muscle. An increased fiber fraction in the P2 state can result in an intracellular Na^+ and water accumulation as shown by ^{23}Na-MRI and ^{1}H-MRI in vivo. Carbonic anhydrase and aldosterone inhibitors shift fibers from P2 to P1, reduce Na^+ and H_2O accumulation, and recover strength.

Expression of sarcolemmal ion channels in slow and fast-twitch muscles of rodents in simulated and actual microgravity

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Sarcolemma ion channels, the expression of which depends on muscle phenotype, are involved in many cellular functions, ranging from control of excitability to modulation of gene expression. Here we report the effects of reduced muscle activity on ion channel expression/function in the slow-twitch soleus muscle in response to simulated (hindlimb-unloading model) and actual (spaceflight) microgravity. Exposure of rats or mice to hindlimb unloading (HU) for 14 days induces a severe atrophy of soleus muscle in parallel to the shift toward a faster phenotype. We showed that the expression/activity of voltage-gated Cl^- (ClC-1) and Na^+ (Nav1.4), and Ca^{2+}-activated K^+ channels was changed in HU Soleus in accord with the phenotypic transition. In addition, although the activity of ATP-dependent K^+ channel (KATP) increased with the phenotypic shift, it was reduced in atrophied fibres, pointing out this channel as a molecular marker of atrophy. The effect of muscle disuse on ion channels was verified in mice exposed to actual microgravity during a 92-days space flight aboard the International Space Station (MDS mission). Preliminary real-time PCR data suggest that expression of ClC-1 and Nav1.4 channels increased in the soleus after actual microgravity exposure as in the HU model. These results show that modulators of ion channels may be useful countermeasures against skeletal muscle dysfunction due to disuse. Supported by Italian Space Agency. The MDS team is gratefully acknowledged.

Regulation of chloride conductance in skeletal muscle

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Cellular excitability is inversely influenced by the passive membrane conductance that in skeletal muscles mainly depends on conductance for Cl^- (GCl). Although myotonic disorders and metabolic poisoning of muscles have shown that GCl can undergo extensive changes, little is known about its regulation under normal conditions. In a series of recent studies, however, we found that GCl is affected by several mechanisms that are likely to be active during muscle activity. Thus, in rat soleus muscles, a CO_2-induced reduction in intra- and extracellular pH to $\sim$6.8 reduced GCl by 47% (from 1730 $\pm$ 150 to 940 $\pm$ 60 $\mu s/cm^2$). Experiments, where only intra- or extracellular pH was reduced showed that the effect was specifically related to the reduction in intracellular pH. Other experiments showed that GCl also could be reduced by addition of lactate ions at normal pH. This effect was maximal at 10 mM lactate where GCl was reduced by 31%. A similar effect of lactate was demonstrated in mechanically skinned fibers. Finally, electrical stimulation led to a $\sim$70% reduction in GCl that followed an exponential decay with a time constant of $\sim$200 APs. This effect was seen on both soleus and EDL muscles but in EDL the reduction in GCl was replaced by an increase to 300% of the initial value when stimulation was continued beyond $\sim$1800 APs. Since acidification, lactate accumulation and excitation are characteristic for active muscles, the results indicate that GCl is substantially regulated during work.

TRPC6 channels are functionally expressed in mouse skeletal muscle and can modulate Ca^{2+} influx and contractile properties during muscle fatigue

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We have recently shown that TRPC6, a member of the transient receptor potential family of cation channels, is expressed in mouse skeletal muscle and localized in the sarcolemma (Krüger et al. Neuromuscul Disord 18:501, 2008). To investigate the functional role of TRPC6, we studied Ca^{2+} influx and intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in isolated interosseus fibres and contractile properties of soleus muscles in response to activators and inhibitors of TRPC6. The application of 25 μM hyperforin, a TRPC6 activator, caused long-lasting increases of $[Ca^{2+}]i$ in the fibres. Pre-incubation with ML-9 significantly attenuated the effect of hyperforin (increase in 340/380 ratio after 260 s: 0.032 $\pm$ 0.018, $n = 9$ with 100 μM ML-9 vs. 0.076 $\pm$ 0.051, $n = 17$ control). Background Ca^{2+} entry, as tested with the Mn^{2+} quench technique, was not affected by ML-9. However, application of 150 μM OAG, a TRPC6 activator stimulated background Ca^{2+} entry by 50% ($n = 65$). Tetanic force of soleus muscles at 10 Hz and 120 Hz stimulation frequency was inhibited by ML-9. Further, during sustained repetitive stimulation (fatigue protocol) force drop to half maximal force occurred 40% faster in the presence of ML-9 compared to control ($n = 8/11$ muscles tested). The data indicate that TRPC6 is functional in mouse skeletal muscle, but does not seem to contribute to background Ca^{2+} entry at rest. We obtained evidence that the channel is involved in fine tuning of Ca^{2+} homeostasis during muscle fatigue.

EC coupling

The sarcoplasmic reticulum and the structural and molecular basis of excitation–contraction coupling

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The sarcoplasmic reticulum (SR) of skeletal muscle cells is a convoluted structure composed of a variety of tubules and cisternae that share a continuous lumen delimited by a single continuous membrane, branching to form a network that surrounds each myofibril. In this network, some specific domains basically represented by the longitudinal SR and the junctional SR can be distinguished. These domains are mainly dedicated to Ca^{2+} homeostasis in relation to regulation of

muscle contraction, with the longitudinal SR representing the sites of Ca^{2+} uptake and storage and the junctional SR representing the sites of Ca^{2+} release. To perform its functions, the SR takes contact with other cellular elements, the sarcolemma, the contractile apparatus and the mitochondria, giving rise to a number of interactions, most of which are still to be defined at the molecular level. We will review recent advancements in understanding the organization of this complex network and of its specific domains.

Calcium release units/mitochondria coupling in developing, ageing and diseased skeletal muscle

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Bi-directional calcium (Ca^{2+}) signaling between mitochondria and intracellular stores (endoplasmic/sarcoplasmic reticulum) underlies important cellular functions, including oxidative ATP production. In striated muscle, this coupling is achieved by mitochondria being located adjacent to Ca^{2+} stores (sarcoplasmic reticulum, SR) and in proximity of release sites (Ca^{2+} release units, CRUs). Specifically, mitochondria are located in pairs between CRUs and Z lines closely associated to the SR on the side opposite to the T-tubules. However, limited information is available with regard to the mechanisms of mitochondrial-SR coupling. Using confocal and electron microscopy/tomography we identified a developmentally-regulated "anchoring system" responsible for orchestrating and maintaining mitochondrial/SR association during postnatal maturation and in adult muscle. Specifically, *mitochondrion-SR coupling* is achieved by small bridges, or *tethers*, linking the outer mitochondrial membrane to the intracellular Ca^{2+} stores of muscle. Mitochondrion-SR coupling provides a structural framework for bi-directional signalling between the two organelles in adult/healthy striated muscle. However, the association between mitochondria and CRUs may be partially (or severely) disrupted in physio-pathological conditions such as ageing and disease, which often results in mitochondria disruption and miss-positioning.

Role of Trisk 32, the 32 kDa triadin isoform, in the calcium homeostasis of skeletal muscle

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Four isoforms of triadins have been identified so far in rat skeletal muscle. While the role of the 95 kDa isoform in excitation–contraction coupling has been studied in detail, the exact function of the 32 kDa isoform (Trisk 32) remains elusive. To understand its role, Trisk 32 was stably overexpressed in L6.G8 myoblasts and the co-localisation of Trisk 32 and the inositol-tris-phosphate receptor (IP3R) shown by immunocytochemistry and co-immunoprecipitation. The functional effects of its overexpression were tested by measuring IP3-mediated Ca^{2+} release induced by the stimulation of bradykinin or vasopressin receptors. The amplitude of the transients evoked by 20 mM bradykinin were significantly higher in Trisk 32-overexpressing ($P < 0.01$; 426 ± 84 nM, $n = 27$) than in control cells (76 ± 12 nM, $n = 23$). The involvement of the ryanodine receptors (RyR) in the bradykinin-evoked Ca^{2+} release was monitored by applying the agonist (30 mM caffeine) and the antagonist (10 mM ryanodine) of the receptor. Under these conditions no significant difference was observed between Trisk 32 overexpressing and control cells. Similarly, store-operated Ca^{2+}-entry was not altered significantly despite the decrease in the expression of STIM1. These results suggest that neither the increased activity of RyRs, nor the amplification of SOCE are responsible for the differences observed in the IP3-mediated Ca^{2+} transients, rather, it was due to the enhanced activity of IP3Rs. Supported by OTKA K75604, NK78398.

Altered myoplasmic calcium dynamics in a transgenic mouse model of Huntington's Disease

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Neurodegeneration in Huntington's Disease (HD) results from an expanded polyglutamine region in the protein huntingtin. The transgenic R6/2 mouse expressing exon 1 of the human huntingtin gene is the most frequently used animal model of HD. In human patients and R6/2 mice mutant huntingtin is also expressed in skeletal muscle and may be involved in causing the atrophic changes found in this tissue. To investigate functional alterations in excitation–contraction coupling, we studied depolarization-dependent Ca^{2+}-transients in isolated muscle fibers of R6/2 mice. Interosseus fibers of R6/2 mice (11–13 weeks old) and of age-matched WT mice were enzymatically dissociated and primary-cultured for up to 2 days. Ca^{2+}-changes elicited by brief electrical shocks causing action potentials or by two-electrode voltage clamping were measured using the fluorescent indicators Fura2 and FuraFF. R6/2 fibers exhibited a significant increase in their mean time constant of relaxation compared to WT fibers. The time course of relaxation in sequences of repetitive activation was fitted using a model to estimate Ca^{2+}-removal by binding and transport. The kinetic changes were found to be compatible with a substantial reduction of both parvalbumin concentration and the Ca^{2+} reuptake rate in R6/2 fibers. Based on this analysis, the peak flux of Ca^{2+} from the sarcoplasmic reticulum was calculated and found to be about half the value in WT fibers. In summary, the results indicate a significant reduction in Ca^{2+} release and removal in skeletal muscle of R6/2 animals transgenic for mutant huntingtin.

Calcium influx analysis by TIRF microscopy on cultured primary myotubes from patients with *RYR1* mutations

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Central core disease (CCD) and malignant hyperthermia (MH) have been linked to point mutations in the gene encoding the skeletal muscle sarcoplasmic reticulum calcium release channel (ryanodine receptor), which is localized on human chromosome 19 (*RYR11*). Central core disease is a relatively mild, slowly progressive autosomal dominant myopathy, characterized histologically by the presence of centrally located cores running the length of the muscle fibres. MH is a pharmacogenetic induced hypermetabolic disease. CCD linked RyR1 mutations are associated with depletion of thapsigarin-sensitive stores and to an increase of the resting calcium level. Influx of Ca^{2+} from the extracellular environment is a major factor influencing the level of the resting intracellular [Ca^{2+}]. Our working hypothesis is that decrease of sarco(endo)plasmic reticulum Ca^{2+} load via leaky ryanodine receptor channels and/or alteration of calcium influx via store operated channels or excitation-coupled Ca^{2+} entry (ECCE), may account for, at least in part, the phenotype of patients with CCD,

including muscle weakness and abnormal secretion of inflammatory cytokines from muscle cells and cells of the immune system. We set out to test the validity of our hypothesis by directly investigating the mechanisms activating calcium influx in myotubes from normal individuals and from patients with CCD and MH by TIRF microscopy. Our data shows that mutations in the RyR1 affect ECCE in human myotubes from CCD and MH patients.

Smooth and striated muscle plasticity

Contractile mechanism and regulation of hypertrophic smooth muscle

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Smooth muscle can adapt its structure and function to altered (patho)physiological demands. One example is the hypertrophy of the urinary bladder in response to urinary outlet obstruction. We have examined this process in rodent models where a partial urethral obstruction is introduced. Following the outlet obstruction the urinary bladder increases in weight >3-times during a period of 1–2 weeks. This pronounced growth is accompanied by cellular hypertrophy, altered expression of contractile proteins and a transition towards a slower, more economical, contractile phenotype. The intermediate filament protein desmin is up regulated, although this change is not required for the hypertrophy. In addition, the influx/release of Ca^{2+} is decreased together with an altered sensitivity of the contractile machinery and changes in the main Ca^{2+} sensitizing pathways (PKC and RhoA/Rhokinase).

Time-resolved analyses of muscle wasting and paralysis in pig and rat experimental ICU models: mechanisms and interventions

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The muscle wasting and impaired muscle function associated with critical illness in ICU patients have negative consequences for recovery from primary disease, but are also the most persistent and debilitating of problems for ICU survivors years after hospital discharge. This study aims at unravelling the mechanisms underlying the severe muscle wasting and muscle paralysis in ICU patients by using unique rat and pig experimental ICU models allowing: (a) analyses of triggering factors, (b) time-resolved analyses of skeletal muscle structure and function, protein synthesis rate, myofibrillar gene/protein expression and intracellular signalling in response to long-term (6 h–2 weeks) post-synaptic block of neuromuscular transmission (NMB), sedation, mechanical ventilation and muscle unloading, and (c) evaluation of intervention strategies. NMB and mechanical ventilation induced a coordinated, but temporally dispersed, activation of different proteolytic pathways involving a rapid transient nuclear translocation of MuRFs, transcriptional repression of myosin synthesis, and myosin loss resulting in atrophy and functional changes at the muscle fiber level. The complete mechanical silencing, absence of weight bearing and no internal strain caused by contractile elements, is forwarded as the most important factor underlying the unique muscle wasting condition and muscle paralysis in ICU patients (acute quadriplegic myopathy or critical illness myopathy). Sepsis and systemic corticosteroid hormone treatment have an additive effect to the mechanical silencing, while NMB per se is of less importance. Two weeks unilateral passive mechanical ankle joint flexion–extension 12 h/day reduced the muscle wasting in distal hind limb muscles by 20%.

Differential phosphorylation of LZ^+/LZ^- MYPT1 isoforms: implications for the regulation of smooth muscle tone

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MLC phosphatase is a trimeric enzyme composed of a catalytic subunit, a 20-kDa subunit of unknown function, and a myosin targeting subunit (MYPT1). During NO stimulation, PKGI mediated phosphorylation of MYPT1 increases MLC phosphatase activity, which produces a decrease in force. Further, alternative mRNA splicing of a 3′ exon produces two MYPT1 isoforms, which differ by the presence or absence of a leucine zipper (LZ^+/LZ^-), and a LZ^+ MYPT1 isoform is required for PKGI induced smooth muscle relaxation. To examine the influence of MYPT1 structure on the ability of PKGI to phosphorylate the protein, we used two MYPT1 fragments, which differed only by the presence ($MYPT1LZ^+$) or absence ($MYPT1LZ^-$) of the LZ. Purified PKGI phosphorylated $MYPT1LZ^+$, but not $MYPT1LZ^-$. Following phosphorylation, $MYPT1LZ^+$ predominantly existed as a di-phosphorylated protein, and mass spectrometry identified S695 and S668 as sites of phosphorylation. These results suggest that MYPT1 structure has an important role in the regulation of vascular tone, and thus differential tissue expression of LZ^+/LZ^- MYPT1 isoforms contributes to the diversity in the sensitivity of smooth muscle to NO mediated vasodilatation.

Is intermittent hypoxia a signal in muscle plasticity?

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Activity influences on muscle phenotype, and the changes in gene expression are dependent on the specific pattern of activity delivered to the muscle. Such changes must rely on signalling pathways being coupled to sensors sensing activity correlates. During activity PO_2 is reduced by an order of magnitude. HIF-1α is a transcription factor that is increased by hypoxia in a pattern specific way, thus intermittent hypoxia is more efficient than continuous hypoxia. We here show that fast muscles have higher levels of HIF-1α and related molecules than slow muscles. When muscles were stimulated with short high frequency trains of action potentials known to induce a fast phenotype the HIF-1α protein level increased, conversely long low frequency trains known to induce a slow phenotype reduced HIF-1α. When HIF-1α was over-expressed in slow fibres after somatic gene transfer in adult mice, a slow-to-fast transformation encompassing oxidative

enzyme, myosin heavy chain, and an increase in calibre was observed. An important role for HIF-1α in regulating human muscle phenotype and performance is supported by genetics since bearers of a hyperactive HIF-1α polymorphism have 50% more fast fibres, moreover this polymorphism is more common in strength athletes. Our data provide a link between muscle activity, hypoxia and muscle phenotype. Fast muscles are a characteristic of two of the most important patient groups namely chronic obstructive pulmonary disease and metabolic syndrome.

Four-and-a-half LIM domain protein 3 regulates MEF2 activity in vitro and is correlated with myosin heavy chain 1 expression in vivo

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Background: The fibre-type ratio is an important contributor to exercise tolerance and is ultimately controlled by the activity of sets of transcription factors (e.g. MEF-2, SRF) and cofactors (e.g. HDACs) acting on fibre-specific promoters. The activity of these transcription factors is controlled by other proteins and we have found that the protein FHL1 correlates with MHC2A expression and can inhibit SRF activity. The related protein FHL3 is highly expressed in skeletal muscle but little is known about and its function. We analysed FHL3 mRNA in the quadriceps of COPD patients who show increased MHC2A and exercise intolerance. We also determined the ability of FHL3 to regulate MEF-2 and HDAC activity in vitro.
Results: FHL3 mRNA was reduced in the quadriceps of COPD patients compared to age-matched controls and tightly correlated with MHC1mRNA. FHL3 bound to HDAC4 and HDAC5 in pull down assays and caused HDAC4-GFP to localise to the cytoplasm in C2C12 cells. Consistent with this observation FHL3 over-expression enhanced the activity of a MEF2 luciferase reporter and reversed the inhibition by HDAC4.
Discussion: These studies identify FHL3 as a novel regulator of MEF-2 activity through the release of HDAC repression, Furthermore the data suggest that the reduction in FHL3 seen in COPD patients may contribute to the MHC switch and their exercise intolerance.

Enhancement of skeletal muscle hypertrophy in heat shock factor 1-overexpressed mice

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Heat stress is one of hypertrophic stimuli for mammalian skeletal muscles. It has been generally accepted that heat stress causes to increase in the expression of heat shock proteins (HSPs) via stress response in skeletal muscles. Although it has been proposed that HSPs has several cellular functions including the protection of cells from various extracellular stressors, physiological roles for HSPs induction and/or stress response in skeletal muscles is still not elucidated. The expression of HSPs is regulated by heat shock factor 1 (HSF1), which binds to heat shock elements located on the upstream region of all Hsp genes. The present study investigated a physiological role of HSF-1-associated stress response in skeletal muscle hypertrophy by using the transgenic mice expressing the active form of HSF1 (Tg-HSF1). Functional overloading on soleus was induced by tenotomy of synergistic muscles, and was maintained for 4 weeks. Increase in protein content of soleus muscles in Tg-HSF1 was enhanced in Tg-HSF1 mice. Results from this study strongly suggested that the HSF1-associated stress response has some physiological role in loading-induced hypertrophy in skeletal muscles. This study was supported, in part, by Grant-in-Aid for Scientific Research (B, 20300218, KG; A, 22240071, TY; S, 19100009, YO) from Japan Society for the Promotion of Science and The Science Research Promotion Fund from The Promotion and Mutual Aid Corporation for Private Schools of Japan (KG).

Cardiac muscle

Determinants of turnover kinetics in vertebrate cardiac myosins

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Loop 1 and residues around the nucleotide binding pocket are likely regulators of the turnover kinetics of myosin heavy chain (*MYHC*). Species-dependent sequence variations in regions near the nucleotide binding pocket may confer different turnover kinetics via interactions with loop 1 to modulate the coordination of MgADP in the nucleotide binding pocket. We screened various *MYHC* genes to identify regions that might account for functional differences between *MYHC* isoforms with sufficient charge density to provide complementary binding for loop 1. One region was indentified (residues 323–351) and revealed that mouse α-*MYHC* expressed Ser rather than the Gly residue at position 341 commonly observed in other mammalian cardiac myosins. We generated mice expressing homozygous S341G alleles for cardiac α-*MYHC* to test the hypothesis that the S341G mutation would reduce ATPase activity by slowing the rate of MgADP release in murine α-*MYHC*, thereby slowing myocardial contraction kinetics. Po and pCa50 did not differ significantly between the WT and S341G skinned myocardium. However, ktr, kdf, and Vo were significantly slower in maximally activated preparations isolated from S341G myocardium. Furthermore, S341G mutant myosin demonstrated a significant reduction in the rate of ADP dissociation. These results indicate that the unique Ser substitution at residue 341 in α-*MYHC* plays a critical role in conferring very fast turnover kinetics in murine myosin. Supported by NIH HL61635.

The molecular mechanisms of thin filament cardiomyopathies: an integrative in silico to in vivo approach

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FHC is a cardiac muscle disorder caused by sarcomeric gene mutations and is the most common identifiable cause of sudden cardiac death in young people. Attempts to link these severe cardiomyopathies to the biophysical changes at the sarcomeric level are limited by the lack of high-resolution structure for portions of the complex and the complexity of protein–protein interactions. To address these issues we developed an integrative approach incorporating

computation, in vitro motility (IVM) and transgenic mouse models. Most FHC mutations in cTnT are within the TNT1 domain, a region unresolved in the extant crystal structure. We used available structural data to develop an atomistic model of the thin filament. Model simulations will help elucidate both the normal TnT modulatory function on tropomyosin, and mutational effects. Along with basic structural questions, we will determine how changes in molecular properties including local flexibility, charge, and accessibility of modulatory domains are altered. A known mutational hotspot is found within a highly charged region of TNT1. Regulated IVM showed Δ160E and E163R mutations disrupt weak actomyosin binding. Reducing ionic strength of the assay rescued function and mutation-specific differences in sliding speed were also observed. This is the first observation of cTnT mutations disrupting weak electrostatic interactions required for strong crossbridge formation. Independent Δ160E and E163R transgenic mouse models exhibited mutation-specific phenotypes that likely represent discrete pathogenic remodeling. These mutations will be studied via both atomistic simulations and a coarse grained model we have developed that should allow simulation of the altered troponin/tropomyosin regulation on a biological timescale and shed light on molecular mechanism.

Beneficial effects of SR33805 in failing rat myocardium

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SR33805, a potent Ca^{2+} channel blocker, has been reported to increase cardiac myofilament Ca^{2+} sensitivity in healthy control rat myocytes when used at 10-5M. Therefore, the present study evaluated the effects of 10-5M SR33805 on contractile properties in ischemic failing hearts after myocardial infarction (MI) in vivo and in vitro. Intact and permeabilized myocytes were used to evaluate the in vitro effects of SR33805 on excitation contraction coupling and myofilaments properties respectively. The in vivo effects were tested after I.P. injection of 20 mg/kg of SR33805. We found that acute application of 10-5M of SR33805 restored the MI-altered cell shortening without affecting the Ca^{2+} transient amplitude. This positive effect of SR33805 is associated by an increase of myofilament Ca^{2+} sensitivity in MI myocytes. In addition, the SR33805-induced sensitization of the myofilaments was associated with changes in the phosphorylation level in two myofilament regulatory proteins: a decrease for troponin I (TnI) and an increase for myosin light chain 2 (MLC-2). Moreover, In vivo acute treatment with SR33805 improved end systolic strain and fractional shortening of MI hearts. The present study indicates that treatment with SR33805 improved cardiac contractility of a failing rat heart by modulating the phosphorylation status of sarcomeric regulatory proteins, which then sensitized the myofilaments to calcium. Our results give also the proof of concept that manipulating specifically the properties of sarcomeric regulatory proteins is a safe way to restore contractile properties in the failing heart.

Slow ATP turnover by myosin in relaxed cardiac fibers: a cardioprotective mechanism in the heart

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Recently Stewart et al. found a new state in skeletal muscles called the "super relaxed state" (SRX), in which the myosin ATP turnover is much lower than the rate in the conventional relaxed state. Here we show that a similar but not identical SRX exists in mammalian cardiac muscle. Quantitative epi-fluorescence was used to measure single ATP turnovers in permeable relaxed rabbit ventricle fibers. Two components of ATP turnover by myosin were found: one with a fast ATP turnover lifetime $\sim$10–15 s, and one with a much slower ATP turnover lifetime 144 ± 10 s. Control experiments showed that the component with the slow lifetime arose from the slow release of nucleotides from a fraction of myosin heads. There was one important difference between the SRX in skeletal and cardiac muscle. In skeletal muscle fibers, a chase with an activating solution results in rapid activation of all myosin heads. In contrast, during a chase with an activating solution in cardiac fibers, myosin heads remain in the super relaxed state. This shows that cooperativity between myosin heads in cardiac thick filaments is reduced relative to skeletal fibers, allowing myosin heads to remain in a super relaxed state while there are adjacent myosin heads present interacting with actin. This observation suggests that the super relaxed state plays a different role in the heart than in skeletal; parking myosin heads in the super relaxed state would be cardio-protective in times of stress such as hypoxia.

Covalent modifications and carbonylation of myofibrillar proteins in human and rat ischemic hearts

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Loss of integrity of troponins (Tn) and/or myofibrillar protein oxidation might explain contractile derangement after myocardial infarction (MI). We investigated their distribution in MI and remote regions of human and experimental rat hearts. Samples were obtained from 16 patients affected with ischemic cardiomyopathy and from 6 unused donors. Western blots analyses showed the presence of degradation and cross-linking of TnT, TnI and desmin only in acute MI regions; immunohistochemistry confirmed this distribution, except for three cases, where changes were detectable in the absence of cell death markers. Myofibrillar protein carbonylation was significantly increased in both MI and remote regions of patients' hearts ($P < 0.04$), and involved TnT. Parallel investigations were performed in 30 rats, exposed to 30 min of regional heart ischemia, relieved or not by reperfusion, and preinjected with pimonidazole to identify the risk zone. Distribution of cross-linked TnT overlapped with that of infarcted cardiomyocytes and involved only a very low number of cardiomyocytes of the risk zone. Protein carbonylation significantly increased in both infarcted and remote heart regions ($P < 0.02$), together with ER stress markers. In conclusion, loss of TnT and desmin integrity was associated only to cardiomyocyte irreversible damage, whereas myofibrillar protein carbonylation extended up to normoperfused regions, representing a possible mechanism of post-ischemic contractile derangement.

Overexpression of cardiomyocyte ribonucleotide reductase (RR) increases contraction without affecting relaxation in adult cardiomyocytes from normal and infarcted hearts

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We previously demonstrated that 2 deoxy-ATP (dATP) increases the magnitude and rate of demembranated myocardial force production at all [Ca^{2+}] via increased cross-bridge binding and cycling rates. We hypothesized that adenoviral overexpression of RR increases cellular [dATP] and improves contraction of normal and failing adult rat cardiomyocytes. At 0.5 Hzstimulation, over-expressing RR significantly increased vs. control (GFP only) rate (122.1 ± 9.9 vs. 82.5 ± 10.6 μm/s) and extent of shortening (10.7 ± 1.0% vs. 6.5 ± 0.8%). Relaxation rate (118.3 ± 30.3 vs. 39.5 ± 10.5 μm/s) and time to 90% relaxation (577 ± 45 ms vs. 804 ± 71 ms) were increased, but 10% and 50% relaxation times did not differ. The rate and extent of intracellular Ca^{2+} release did not differ, but the rate of Ca^{2+} re-uptake increased with RR (measured by Fura-2). Interestingly, viral mediated expression of constitutively active RRM1 (D57N) similarly increased the rate and extent of shortening, but decreased Ca^{2+} transient amplitude. Similar differences were observed between all groups at 1 and 2 Hz stimulation. Adult cardiomyocytes from infarcted hearts had significantly impaired rate and extent of shortening compared to cardiomyocytes from normal hearts, and this depressed function was rescued by overexpression of RR. These data suggest direct targeting of crossbridge cycling can enhance cardiac contractility without impairing relaxation in normal and infarcted hearts. HL091368 (MR), AHA2310117 (FSK).

Stem cells and regeneration

Cell fate switch between cardiac and skeletal muscle lineages

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Stem and progenitor cells that reside in the post-natal heart are extensively studied as a potential therapeutic tool for cardiac diseases. While surface marker expression and ability to generate cardiomyocytes have been characterized in some detail for several types of these progenitors (Galvezet et al. 2008; Yi et al. 2010), little is known about how cardiac differentiation is regulated. Recent works have highlighted an important role of microRNAs, often encoded in the introns of structural muscle genes, in modulating expression of myogenic transcription factors and thereby regulating cardiac and skeletal myogenesis. In the present study we investigated the role of cardiac progenitors in β sarcoglycan null (Scgb null) mouse, a model for limb girdle muscular dystrophy type 2E that undergo a progressive dilated cardiomyopathy. We isolated and cloned cardiac progenitors on the basis of different stem cell markers (Islet-1, cKit, Sca1 and AP) from Scgb null mouse hearts. Dystrophic cardiac progenitors show an altered miRNA expression profile, and spontaneously differentiate into skeletal muscle fibers both in vitro and when transplanted into regenerating muscles or infarcted hearts. These findings provide new insights on the fate choice of mesodermal progenitors and makes the task of utilizing endogenous cardiac stem cells for therapeutic approaches more complex for certain genetic disease.

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MGF a splice variant of the IGF-I gene that is expressed after exercise and tissue damage activates human muscle satellite (progenitor) cells

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Muscle fibres when subjected to mechanical strain produce a growth/repair factor that has been called MGF (Mechano Growth Factor). This is derived by splicing of the IGF-I gene and in the human involves a 49 base insert that results in a reading frame shift and a unique C terminal peptide which initiates activation of the satellite cells. Previous work has shown that older muscles are less able to produce MGF therefore the question was, can the satellite cells which involves several stages, be activated in older muscles by MGF? It was found that the unique MGF E domain with the 24aa C terminal peptide significantly increased the proliferative life span of satellite cells in primary cultures from neonatal human and young adult muscle but not in older adult muscle. However, when using differentiated cultures, the myotube size and growth potential was assessed, this was actually higher in the older muscle due to a decreased in the percentage of "Reserve Cells" (unfused myoblasts). These data indicate that as we age MGF is a rate limiting factor in muscle repair and adaptation but may be effective if administered.

iPS-derived muscle progenitors: how many needles in the haystack?

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Induced pluripotent stem cells (iPS) are generated from somatic or adult stem cells and hold great potential for regenerative medicine and drug screening. In vivo iPS-driven muscle regeneration could gather clinical interests, even though several concerns, e.g. teratoma risk, must be addressed and overcome. Here we show that murine mesoangioblasts (MABs) can be isolated by flow citometry and reprogrammed to pluripotent cells (MAB-iPS) by *Oct4/Sox2/Klf4/cMyc* retroviral overexpression. MAB-iPS are able to induce mature teratomas, which are constituted by ≥70% skeletal muscle myoblasts. Following a transient transfection of *Pax3* and *Pax7*, MAB-iPS robustly undergo spontaneous in vitro myotube formation and yield sortable CD56+ progenitors, with higher efficiencies than fibroblast-derived iPS. Once injected intramuscularly into dystrophic α *sarcoglycan-null* mice, these progenitors contributed to up to 50% fibre regeneration and α *sarcoglycan* expression, without any teratoma

formation. Moreover, they partially reconstituted the endogenous satellite cell pool. Although very promising, these results just corroborate a newly starting perspective of antigen-specific and clinically relevant isolation of iPS-derived myogenic progenitors.

Amniotic fluid stem cells integrate into muscle satellite cells niche

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Spinal muscular atrophy (SMA) is a recessive neuromuscular disorder; using a Cre recombinase placed under the control of the human alpha-skeletal actin promoter, mice carrying homozygous deletion of Smn to skeletal muscle (*HSA-Cre, SmnF7/F7* mice) were engineered and used in the study. GFP+ Amniotic Fluid Stem (AFS) cells have been systemically injected in transgenic mice to test the in vivo myogenic potential and to investigate if these cells could be considered a good source for the treatment of muscle-related diseases. One month after transplantation, muscles from AFS cells treated mice displayed normal morphological appearance with very low number of regenerating myofibers (<1%) and physiological dystrophin expression. Moreover, about 37.49 ± 0.97% of fibers were GFP+ and PCR analyses highlighted the presence of GFP on different muscles. Conversely, the untreated mice possessed high number of centri-nucleated fibers (about 63.70 ± 0.41%) and had a down regulation of dystrophin expression. Physiological analyses on treated mice at 1 month of injection underlined the positive effect of AFS cells transplantation showing that these mice recovered more than 75% of force compared to the untreated. Tibialis underwent cardiotoxin injection one month after AFS cells transplantation and 15 days later the cells were able to generate new fibers further proving their functional integration. Finally, secondary transplants were performed injecting stripped satellite cells derived from primary transplanted mice directly into muscle. One month later 33.09 ± 0.99% fibers were found to be GFP+. These results proved that AFS cells are able to repopulate the depauperated muscle niche becoming new satellite cells themselves.

Outcome measures validation study for mesoangioblasts transplantation in children affected by Duchenne Muscular Dystrophy

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The aim of this study is to establish a reliable tool of reproducible assessment of muscle strength in children affected by Duchenne muscular dystrophy (DMD) which will be selected for mesoangioblasts transplantation. We have developed a potential treatment for DMD based on infusion of cells from a healthy donor capable. This is a single centre, prospective, non-randomised, study of validation of outcome measures on 30 ambulant patients aged 5–12 years old affected by DMD including a cohort of 15 healthy aged matched males. We perform 2 days evaluation each 3 month for 1 year. During each assessment the following outcome measures are applied to DMD subjects: North Star Scale and 6 min walking test during the first day; quantitative assessment using the Kin Com 125 machine during the second day. The controls subjects will perform quantitative assessment twice in a year. We divided the patients into three subgroups of age (5–7, 8–9, 10–12 years). The results of this preliminary part of the study show specific correlation between functional and quantitative tests in stronger children. Kin Com measurements correlate appropriately with functional tests for 10–12 years old DMD boys, while show a major variability in muscle strength for 8–9 years old DMD boys. This preliminary study demonstrates that our assessment may represent a useful tool to monitor the progress of DMD in ambulant children to determine the pre-transplantation story of the children who will be later treated with mesoangioblasts.

Muscle and exercise

Is neuronal nitric oxide synthase (nNOS) involved in exercise-induced mitochondrial biogenesis and angiogenesis in skeletal muscle?

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At the sarcolemma of skeletal muscle fibers, high concentrations of nitric oxide (NO) are produced by neuronal NO synthase (nNOS). Due to its localization and its established function as signaling-involved enzyme, we hypothesize that nNOS plays a role in the induction of mitochondrial biogenesis and angiogenesis in skeletal muscle in response to endurance exercise. Tibialis anterior muscles (TA) were collected from cohorts of C57BL/6-mice undergoing treadmill exercise for 5 weeks or remaining sedentary ($n = 8$ mice each). Real-time PCR analysis and quantitative immunoblotting revealed nNOS to be significantly more expressed ($P \leq 0.05$) in TA of trained than untrained mice at the mRNA (+120%) and protein (+80%) level. Correspondingly, vastus lateralis muscle (VL) biopsies of 10 sedentary male subjects collected before and after a moderate training (4 × 30 min jogging per week; 6 months) contained significantly ($P \leq 0.05$) higher (+34%) nNOS mRNA levels after exercise which correlated with training-induced raises in subsarcolemmal mitochondrial density and capillarity (r = 0.3–0.7). Catalytic activities gained by histochemistry on cryosections (succinate dehydrogenase (SDH) and nNOS-specific NADPH diaphorase activity) demonstrated a positive correlation between SDH and nNOS expression in type I and type IIa fibers before and after exercise (r = 0.3–0.8). These correlation data support the hypothesis nNOS is involved in skeletal muscle plasticity in response to exercise.

Effect of strength training, strength training combined with a greater intake of proteins and gender influence on contractile performance and MHC isoform composition in human upper limb muscle fibres

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Strength or resistance training is frequently used to improve muscle mass and performance. Although there are many studies on the

relation between training protocols and fibre adaptations in lower limb muscles, there are relatively few data available in upper limb muscles. This study aimed to analyse at muscle fibre level the effect strength training in 18 healthy subjects (9 men and 9 women) who trained three times a week for 2 months and were further divided in two groups assuming different amount of proteins. Needle biopsy samples were collected from upper limb muscle (Longissimus Dorsi). Single muscle fibres were dissected and the following parameters were determined: cross sectional area (CSA), isometric tension, i.e. isometric force/CSA during maximal activations (Po), and MHC isoforms composition. The mechanical and molecular parameters were compared to investigate pre and post-training differences and to understand whether the response of single muscle fibres was affected by different protein intake or gender. The results obtained showed that (1) CSA increased significantly, although large variations in CSA increase were detectable among different subjects, without any clear relation with the protein intake. Surprisingly, only men fibres showed significant increases in CSA, (2) isometric tension (Po) increased significantly, i.e. the increase in force was greater than the increase in CSA, (3) a trend towards a MHC transition from fast to slow was detectable.

Effects of muscle cooling on force generation capacity in older and young healthy men

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The purpose of this study was to investigate the effects of muscle cooling on force generation capacity in older and young male individuals. Nine older (73.8 + 4.2 years) and 8 young (24.6 + 3.0 years) volunteered to take part in this study which was approved by the local ethical committee. All subjects attended to one experimental session involving the plantar-flexors muscle group of the right leg. Muscle temperature was monitored by inserting a probe (Ellab ltd, Norfolk, UK) into the lateral gastrocnemius muscle (GL) about 1 cm under the fascia. Subjects were tested for maximal voluntary isometric contractions (MVC), elicited muscle twitch contractions and % of voluntary activation (twitch interpolated technique) at two muscle temperatures: control ($\approx$34–35°C) and cold ($\approx$29–30°C). Cooling was induced by wrapping the leg in a cuff where water was circulated at a T of 5°C. Bipolar surface EMG was recorded during the MVC from both the GL and the tibialis anterior muscles. MVC and % of voluntary activation and EMG responses, although lower in older than young individuals ($P < 0.05$), were not affected by cooling. Muscle twitch responses, time-to-peak and the half-relaxation-time were prolonged in older ($P < 0.001$) and affected by cooling in both groups ($P < 0.05$), whereas the twitch torque was not different in all conditions. In conclusion, although cooling did not modify both maximal force and activation capacity it significantly slowed down the muscle contractile properties. This could have important implications for the older individual especially in relation to maintaining balance in response to a perturbation.

Bed rest impairs skeletal muscle oxidative function independently from constraints related to cardiovascular O_2 delivery

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A functional evaluation of skeletal muscle oxidative function was performed during one-legged knee-extension (KE) incremental exercises following a 35-day bed-rest (BR) (Valdoltra 2008 BR campaign). Ten young male volunteers were evaluated. Pulmonary gas exchange, cardiac output (Q') (by impedance cardiography), vastus lateralis muscle O2 extraction (by near-infrared spectroscopy) were determined. Data obtained during KE were compared to those obtained during cycloergometric exercise (CE) (Porcelli et al. 2010) after the same bed rest period (Valdoltra 2007 BR campaign). Peak O2 uptake (V′O2peak; L/min) during KE decreased by $\sim$17% after (0.99 ± 0.05 [mean $\pm$ SE]) vs. before BR (1.23 ± 0.08); percentage-wise, this decrease was very similar to that described during CE (2.63 ± 0.11 vs. 3.20 ± 0.18). During KE, peak Q' (17.8 ± 1.1 l/min before vs. 16.1 ± 0.5 after BR) was unaffected by BR; values were significantly lower than those obtained during CE (26.5 ± 0.5 before vs. 21.4 ± 0.7 after BR); this confirms that KE did not represent a maximal burden for the cardiovascular system. Peak skeletal muscle O2 extraction was significantly lower after vs. before BR, both during KE (45 ± 4% vs. 64 ± 4) and CE (41 ± 3 vs. 63 ± 3). The model by di Prampero (2003) was applied to KE data and confirmed a much higher role of skeletal muscle factors ($\sim$70%) than cardiovascular O_2 delivery ($\sim$30%) in limiting $V'O_2max$. After eliminating, by the adopted KE protocol, constraints related to cardiovascular O_2 delivery, the very similar relative decreases in $V'O_2$ peak and skeletal muscle peak capacity of O_2 extraction after 35 days of BR suggest that the latter determines a substantial impairment of skeletal muscle oxidative function. Financial support by ASI–OSMA Contract I/007/06/0, Workpackage 1B-31-1 is acknowledged.

Effects of training on IL-6 and the role of antioxidant supplementation

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Antioxidant supplementation has been shown to attenuate the acute exercise-induced increase in plasma IL-6. Here, we studied the effect of antioxidants on the regulation of IL-6 in muscle and circulation in response to acute exercise before and after intense endurance training. Twenty-one young healthy men were allocated into either an antioxidant (AO; vitamin C and E) or a placebo (PL) group. A 1 h-acute bicycling exercise trial at 65% of maximal power output (P_{max}) was performed before and after 12 weeks of progressive endurance training. The acute exercise-induced plasma IL-6 was blunted after the training period in the PL group ($P < 0.05$) but not in the AO group. Endurance training lowered resting levels and attenuated the acute exercise-induced increase in muscle-IL-6 mRNA in both groups. There was an overall increase in oxidative stress as evaluated by malondialdehyde (MDA) in skeletal muscle after training in the AO group compared to placebo ($P < 0.05$). This was accompanied by a general increase in skeletal muscle mRNA expression of antioxidative enzymes, including catalase (CAT), copper-zinc superoxide

dismutase (CuZnSOD) and glutathione peroxidase 1 (GPX1) mRNA expression in the AO group. However, skeletal muscle protein contents of catalase, CuZnSOD, or GPX1 were not affected by training or supplementation. In conclusion, our results indicate that vitamin C and E supplementation prior to endurance training blunts the training-induced decrease in plasma IL-6 in response to exercise, possibly through increased oxidative stress during acute exercise after training.

Regulation of muscle protein synthesis (MPS) by resistance exercise (RE), hypoxia and amino acids (AA)

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We have investigated the regulation of MPS by exercise, hypoxia and AA. First we tested the effects of hypoxia on MPS after RE in young men. MPS increased significantly 2.5 h after RE in normoxia (0.033 vs. $0.104\%h^{-1}$) but was unaltered in the rest leg ($0.043\%^{-1}$) or the exercised leg after 3.5 h of hypoxia ($0.06\%h^{-1}$) for which blunting correlated with extant SpO_2 ($r^2 = 0.48$). Thus, systemic hypoxia blunts increases in MPS after RE. Second we tested how the rise-and-fall of MPS occurs after feeding in young men. MPS rate trebled 45–90 min after 48 g whey (0.03 ± 0.01 vs. $0.1 \pm 0.01\%h^{-1}$) thereafter falling to baseline despite raised plasma/muscle [Leu] (+105/+54%) and persisting anabolic signalling at 180 min. At this time, eIF2α phosphorylation was induced. Thus AA and anabolic signals outlast MPS responses perhaps due to excess AA evoking an unfolded protein response. Third we tested whether, as for eccentric contractions, Leu-induced increases in S6K1 signalling are phosphatidic acid (PA)-dependent. Myotubes were contracted or incubated with Leu (±)1-butanol (PA inhibitor). Increases in S6K1 phosphorylation (+53%) and MPS after contraction were ablated by 1-butanol whereas increases by Leu (+33%) were unaffected. Thus, Leu and contraction regulate S6K1 separately, perhaps explaining why Leu and contraction synergistically stimulate MPS in vivo. Finally, how ageing affects temporal responses to feeding and chronic responses to training will be considered.

Muscle growth and hypertrophy

Regulation of muscle growth and homeostasis in ageing and disease

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Homeostasis represents one of the most important and critical parameter of adult skeletal muscle and it is defined as the capability of a system to maintain a constant state of complexity and order in a dynamic equilibrium. Most muscle pathologies are characterized by the progressive loss of muscle tissue due to chronic degeneration combined with the inability of regeneration machinery to replace damage muscle tissue. The persistent protein degradation observed in muscle diseases reflects a pathological muscle catabolism, known as muscle wasting. The continual synthesis and degradation of cell proteins is the result of normal intracellular metabolism and represents an important homeostatic function of muscle tissue. Altering the homeostatic set point is detrimental for myofibers survival and muscle functional integrity. Aging and genetic diseases, such as muscular dystrophies and amyotrophic lateral sclerosis are characterized by alterations in metabolic and physiological parameters, progressive weakness in specific muscle groups, modulation in muscle-specific transcriptional mechanisms and persistent protein degradation. The inability to regenerate and repair the injured muscle is another serious complication in muscle pathologies. Although considerable information has accumulated regarding the physiopathology of muscle diseases, the associated molecular mechanisms are still poorly understood. Among growth factors, the *insulin-like growth factors 1* (*IGF-1*) has been implicated in many anabolic pathways in skeletal muscle, where it plays a central role during muscle regeneration and it has been considered a promising therapeutic agent in staving off advancing muscle weakness during ageing and in several muscle diseases. In this presentation, the roles of *IGF-1* isoforms, IL-6 and oxidative stress on muscle growth and homeostasis, along with the potential therapeutic role of local *IGF-1* isoform on muscle aging and diseases, will be discussed.

Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation

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A better understanding of the signalling pathways that control muscle growth is required to identify appropriate countermeasures to prevent or reverse the loss of muscle mass and force induced by aging, disuse, or neuromuscular diseases. However, two major issues in this field have not yet been fully addressed. The first concerns the pathways involved in leading to physiological changes in muscle size. Muscle hypertrophy based on perturbations of specific signalling pathways is either characterized by impaired force generation, e.g., myostatin knockout, or incompletely studied from the physiological point of view, e.g., IGF-1 overexpression. A second issue is whether satellite cell proliferation and incorporation into growing muscle fibers is required for a functional hypertrophy. To address these issues, we used an inducible transgenic model of muscle hypertrophy by short-term Akt activation in adult skeletal muscle. In this model, Akt activation for 3 week was followed by marked hypertrophy (50% of muscle mass) and by increased force generation, as determined in vivo by ankle plantar flexor stimulation, ex vivo in intact isolated diaphragm strips, and in single-skinned muscle fibers. No changes in fiber-type distribution and resistance to fatigue were detectable. Bromodeoxyuridine incorporation experiments showed that Akt-dependent muscle hypertrophy was accompanied by proliferation of interstitial cells but not by satellite cell activation and new myonuclei incorporation, pointing to an increase in myonuclear domain size. We can conclude that during a fast hypertrophic growth myonuclear domain can increase without compromising muscle performance.

Myonuclei acquired by overload-exercise are permanent and precede hypertrophy

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Effects of previous strength training can be long lived even after prolonged subsequent inactivity, and retraining is strongly facilitated by a previous training episode. Traditionally such "muscle memory" has been attributed to neural factors in the absence of any identified local memory mechanism in muscle tissue. It has generally been assumed that myonuclei are lost by selective apoptosis of some nuclei during atrophy, while new nuclei are added from satellite cells during hypertrophy. We have used in vivo staining techniques to study the time-course of overload-hypertrophy and subsequent atrophy. The study revealed a 50% increase in the number of nuclei after 6–10 days of overload. This increase preceded the onset of hypertrophy by 2–3-days. The new nuclei are retained during severe atrophy caused by subsequent denervation lasting for a considerable period of the animal life span. We also show that atrophy is attenuated in fibers that has been overloaded before denervation, compared to those that has only been denervated. The permanence of myonuclei can therefore possibly serve as a cell-biological substrate for "muscle memory". Since the ability to create myonuclei is impaired in the elderly, individuals may benefit from strength training at early age. Also, as anabolic steroids facilitate more myonuclei, nuclear permanency may also have implications for exclusion periods after a doping offence.

β_2-adrenergic agonists reactivate ERK1/2 and Akt signaling and suppress atrophic genes in skeletal muscles from fasted mice

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This study was undertaken to investigate the short-term effects of β_2-adrenergic agonists (BA) on ERK1/2 and Akt signaling and Ub-ligases expression in skeletal muscles during fasting. For that, muscles from fed and 2-days fasted C57Bl6/J mice were analyzed after 1, 4, and 12 h of a single s.c. injection of Clenbuterol (CB; 3.000 μg/kg) or Formoterol (FOR; 30, 300, and 3.000 μg/kg). Gene expression (Atrogin-1 and MuRF1) was quantified by RT-qPCR. The phosphorylation (p) status of ERK1/2 and Akt was assessed by Western blotting. cAMP levels were measured by immunoenzymatic assay. Fasting reduced mass (10%), cAMP levels (36%), pERK1/2 (~40%), and pAkt (~80%) in muscle. Moreover, mRNA expression of Atrogin-1 (10-fold) and MuRF1 (30-fold) were increased. At 1 h, BA reestablished cAMP levels and pERK1/2 and increased (~4-fold) pAkt in atrophied muscles. FOR induced similar effects in a dose 100-fold lower than CB. The stimulatory action of CB and FOR on kinases lasted 4 and 1 h, respectively. Atrogin-1 expression was completely suppressed 4 h after FOR and reduced (~70%) 1 h after CB, remaining low until 12 h. MuRF1 expression showed the same profile than Atrogin-1 after FOR and decreased (55%) only 12 h after CB. Taken together, these data indicate that the short-term treatment with BA is able to reactivate, possibly via cAMP pathway, the ERK1/2 and Akt signaling and suppress the expression of Ub-ligases during fasting, being the FOR more efficient than the CB. Supported by FAPESP & CNPq.

Muscle atrophy

MuRF1 is a muscle fiber-type II factor and combined with MuRF2 regulates type-II fiber trophicity and specification

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MuRF1 is a member of the RBCC (RING, B-box, coiled-coil) superfamily that has been proposed to act as an atrogin during muscle wasting. Here, we show that MuRF1 is specifically expressed and preferentially induced in type-II muscle fibers after denervation. Analysis by immunofluorescence shows that after 14 days of denervation, MuRF1 protein was further elevated but remained preferentially expressed in type-II muscle fibers. Consistent with a fiber-type dependent function of MuRF1, the tibialis anterior muscle (rich in type-II muscle fibers) was highly protected against atrophy (only 5% loss vs. WT) in MuRF1-KO mice when compared to the soleus muscle with mixed fiber-types (35% loss vs. WT). We also determined fiber-type distributions in MuRF1/MuRF2 double-deficient KO (dKO) mice, because MuRF2 is a close homolog of MuRF1. MuRF1/MuRF2 dKO mice showed a profound loss (22% of reduction) of type-II fibers in soleus muscle. In summary, our data suggest that expression of MuRF1 is required for remodeling of type-II fibers under pathophysiological stress states. Furthermore MuRF1 and MuRF2 together seem to be required for maintenance of type-II fibers. Financial Support: FAPESP, CNPq, DFG.

Developmental regulation of MuRF ubiquitin ligases during myofibril assembly and turnover

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MuRF2 belongs to the RING-finger, B-box, Coiled-Coil (RBCC) family of putative E3 ligases, whose members are implicated in ubiquitination and signal transduction. Three genetically distinct MuRFs (1, 2 and 3) are known, of which MuRF2 is the least well characterised. Several observations suggest MuRF2 might be involved in cardiac myofibrillogenesis. Firstly, in humans it is expressed as four isoforms—the smallest 27 kDa variant is cardiac-specific. Secondly, MuRF2 transcripts are developmentally down-regulated. Thirdly, MuRF2 associates transiently with microtubules, myosin and titin during sarcomere assembly. Investigation of the expression profile of MuRF2 in the mouse heart shows that it is expressed as at least three isoforms of size 60, 50 and 27 kDa. In embryonic and early postnatal stages, the 50 kDa isoform is more prominent than its 60 kDa counterpart, whereas in adults, the situation is reversed. This isoform switch occurs postnatally, coinciding with the onset of the exclusively hypertrophic growth phase in the myocardium and associated switches in many other sarcomeric protein isoforms. The developmental expression of MuRF2 is also tightly linked to that of other proteins implicated in muscle atrophy and turnover. Surprisingly, MuRF2 knockouts have no overt cardiac or skeletal muscle phenotype, suggesting a degree of compensation by other MuRFs, but to what extent such cooperation is afforded in vivo is unknown. Isoform-specific knockdown of MuRF2 in cardiomyocytes and skeletal muscle cell lines leads to alterations in microtubule

dynamics and myogenic differentiation. These observations hint that MuRF2 isoforms play an important role in myofibrillogenesis and myofibril turnover.

Genomics, proteomics and epigenetics

Signal-dependent epigenetic control of endogenous muscle regeneration

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Deciphering the intracellular signalling that converts the inflammatory cues released in the regenerative environment into the epigenetic modifications that control gene expression in adult stem cells is a key issue in regenerative medicine. We have dissected key components of the p38 signalling, which is elicited by muscle regeneration cues, to the chromatin muscle stem cells (satellite cells). Our studies have identified different chromatin targets of the regeneration-activated p38 signalling, that coordinate activation (SWI/SNF chromatin remodelling complex) and repression (Polycomb repressive complex (PRC) of distinct subsets of genes during satellite cell-mediated regeneration of normal and dystrophic muscles. We have also identified a novel population of interstitial muscle cells that cooperate with satellite cells to promote regeneration of injured muscles. We will present our latest evidence on the mechanism underlying functional interactions between satellite and interstitial muscle cells, and the pharmacological interventions by which we can manipulate these interactions with epigenetic drugs, such as histone deacetylase inhibitors (HDACi) and compounds that inhibit the enzymatic component of the PRC2 complex—the histone methyltransferase Enhancer of Zeste 2 (EzH2).

MicroRNA control of quiescence in skeletal muscle stem cell

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Satellite cells are skeletal muscle stem cells that can undergo self-renewal and differentiation in the regeneration process after the activation from their quiescent state. Quiescente Satellite Cells (QSC) are localized in their niche, under the basal lamina, along a muscle fiber. The QSC regulation is critical to sustain stem cell function trought life. QSC are dormant cells but they are ready to respond quickly and to activate to proper signals. MicroRNAs (MiRNAs) are small non coding RNAs known to regulate gene expression at post transcriptional level. In particular, their function is now recognized to work as a balancing act between different pathways, serving as nodes of signalling networks and affecting responsiveness of cell to signalling molecules in stem cells. We identified the miRNA653 as a quiescente specific miRNA. In QSC, miRNA653 is required, ex vivo and in vivo, to modulate the cell activation and the re-entering into the cell cycle. Targeting several transcription factors important to play a role in the myogenic program, miRNA653 is able to keep the cell in a steady state but, upon its withdrawal, the QSC become quickly activated. Our results therefore define a role of miR653 in adult muscle stem cell biology and tissue repair.

Regulation of cytosolic muscle protein degradation in *C. elegans*

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Muscle atrophy severely affects health, being clinically associated with fasting, chronic malnutrition, disuse, denervation, sepsis, cancer, diabetes, renal failure, and heart failure. Over the last 10 years we have developed *C. elegans* as a model for identifying genes regulating cytosolic muscle protein degradation and for analyzing the signalling networks that regulate such degradation. Currently we know at least five extra-muscular signals regulate at least three distinct intramuscular proteolytic mechanisms. We selected 237 genes, previously shown by mutation to give an altered muscle phenotype, and have screened 162 for altered muscle protein degradation, synthesis and/or dystrophy using RNAi, mutants and our established transgenic lines and methods. RNAi results show that 41 genes appear to regulate myofibre morphology, 43 mitochondrial morphology and 49 cytosolic muscle protein degradation. Of these, 40 genes regulate a single compartment, 3 genes regulate the myofibers and mitochondria, 7 myofibers and cytosol, 3 mitochondria and cytosol, and 16 all three compartments. Despite limitations, these data suggest that RNAi in *C.elegans* is an efficient tool to widen our understanding of muscle protein degradation. We now aim to determine if newly identified genes regulate cytosolic muscle protein degradation via established or new regulatory pathways/mechanisms. This work is funded by NIH NIAMS.

PGC-1α and PGC-1β regulate protein synthesis in C2C12 myotubes

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Skeletal muscle atrophy is characterised by increased rates of protein degradation and/or decreased rates of protein synthesis. Overexpression of peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α) or PGC-1β can attenuate muscle atrophy, and this has been attributed to a decrease in protein degradation. This study investigated the role of PGC-1α and PGC-1β in protein synthesis in C2C12 myotubes. Myotubes were infected with GFP, PGC-1α, or PGC-1β adenoviruses, and protein synthesis was measured at basal levels and following dexamethasone (DEX) treatment. PGC-1α or PGC-1β overexpression resulted in a 25–28% increase in protein synthesis. DEX decreased protein synthesis by 15% in the GFP-infected myotubes. However, overexpression of PGC-1α or PGC-1β was able to prevent the DEX-induced decrease. Treatment with LY294002, an inhibitor of PI3K/Akt, did not prevent the PGC-1α or PGC-1β driven increase in protein synthesis. This effect was therefore independent of Akt, a major kinase involved in muscle growth. Another potential mechanism for the PGC-1α and PGC-1β driven increase in protein synthesis may be via their regulation of microRNAs (miRNAs). The expression of miR-1 and miR133a, two miRNAs thought to play a role in muscle hypertrophy, were downregulated by PGC-1α and PGC-1β. Further studies will determine if these two miRNAs are directly involved in the PGC-1α and PGC-1β regulation of protein synthesis.

Genetic approaches to study the role of cripto in muscle regeneration and development

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Increasing evidence indicate that the mechanisms involved in embryogenesis also function postnatally in tissue repair and regeneration, when aberrantly activated, they may contribute to cancer progression. In this scenario, the EGF−CFC cripto gene turned out to have a crucial role. Cripto is a key player both in embryogenesis and in embryonic stem cells. Up to date, Cripto re-expression in the adult was restricted to tumorigenesis. We have recently shown that Cripto is re-expressed in skeletal muscle regeneration and that viral -mediated Cripto overexpression accelerates muscle regeneration in vivo. To study the biological role of Cripto on muscle development and regeneration, we are generating Cripto gain of function and loss of function mouse models, using the Cre-lox strategy. To this end, we produced a recombinant vector carrying the RedFP, flanked by loxP sites, upstream of a Cripto-ires-GFP cassette, which is transcribed upon activation of CRE recombinase. We first assessed the activity of the recombinant vector, lox-CriptoiresGFP, in 293 cells and showed that Cripto and GFP were expressed upon cotransfection with a Cre expressing vector. Transgenic mice carrying the lox-CriptoiresGFP construct have been generated and are being characterized. To overcome the embryonic lethality of Cripto KO mice, we used cripto flox/fox mutants, crossed with a transgenic line carrying the Tamoxifen-inducible Cre recombinase. These mouse models, by allowing the fine modulation of cripto in vivo, will be instrumental to unravel its role in skeletal muscles.

Striated muscle disease

Antisense oligonucleotides to induce exon 51 skipping in boys with Duchenne muscular dystrophy: what we are learning from clinical trials

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The UK MDEX Consortium (http://www.mdex.org.uk/) has been involved since 2005 in preclinical studies and in clinical trials using antisense oligonucleotides to induce exon skipping in boys with Duchenne muscular dystrophy (DMD). In close collaboration with AVI Biopharma, in 2008 we completed a two doses-escalation study of a morpholino oligonucleotide (AVI-4658) which induces skipping exon 51 in dystrophin mRNA in seven patients with DMD. In 2009 we have started and now almost completed, also in close collaboration with AVI Biopharma, an open label, dose escalation study in ambulant DMD boys aged 5–15 years with deletions that benefit from skipping exon 51. This study consists of 12 weekly administrations of AVI-4658 followed by a muscle biopsy to assess dystrophin expression at baseline and 14 weeks. During the last few years we have optimised methodologies for the identification of lead antisense compounds; we have elected to skip exon 51 based on our experience of the mild resulting in-frame mutations; we have developed techniques to detect and quantitate small changes in dystrophin protein expression, and of its associated protein partners in the dystrophin glycoprotein complex. The encouraging results of the previous IM study, and the interim analysis of the ongoing systemic IV study, together with the excellent tolerability profile of the morpholino antisense oligonucleotides, clearly suggest that this approach has the potential to lead to the development of a drug that could play a role in the treatment of DMD. A number of challenges remain, ranging from the optimal administration regimens of the antisense oligonucleotides, to the initiation of studies targeting other exons, to the validation and adoption by the international scientific community of clinical and biochemical outcome measures which could allow to compare and learn from the different studies which are being planned or executed, to the regulatory complexity of antisense oligonucleotides as indeed the level of personalised approach that these compounds bring is currently without precedent for a genetic disease. In my presentation will discuss what we have learned since 2005 in relations to these various issues and how this is informing our future approach to exon skipping in DMD.

Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype

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M1 macrophages play a major role in worsening muscle injury in the mdx mouse model of Duchenne Muscular Dystrophy. However, mdx muscle also contains M2c macrophages that can promote tissue repair, indicating that factors regulating the balance between M1 and M2c phenotypes could influence the severity of the disease. Because IL-10 modulates macrophage activation in vitro and its expression is elevated in mdx muscles, we tested whether IL-10 influenced macrophage phenotype in mdx muscle and whether changes in IL-10 expression affected muscular dystrophy. We found that ablation of IL-10 expression in mdx mice increased muscle damage in vivo and reduced mouse strength. Treating mdx muscle macrophages with IL-10 reduced activation of the M1 phenotype, assessed by iNOS expression, and macrophages from IL-10 null mutant mice were more cytolytic. We also found that muscle cells in mdx muscle expressed the IL-10 receptor, suggesting that IL-10 could have direct effects on muscle cells. We assayed whether ablation of IL-10 in mdx mice affected satellite cell numbers, using Pax7 expression as an index, but found no effect on Pax7 expression. However, IL-10 mutation significantly increased myogenin expression in vivo during the acute and the regenerative phase of mdx pathology. Together, the results show that IL-10 plays a significant regulatory role in muscular dystrophy by reducing M1 macrophage activation and cytotoxicity, and by modulating satellite cell differentiation.

Molecular pathology of SERCA1 mutants: cattle congenital pseudomyotonia as an animal model for investigating human Brody disease

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Brody disease is a rare inherited disorder of skeletal muscle due to a sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) deficiency, resulting from a defect of ATP2A1 gene coding for SERCA1 isoform. The SERCA1 isoform, expressed in fast-twitch (type 2) skeletal muscle fibers, is a key participant in the Ca^{2+} homeostasis, being responsible for the transport of Ca^{2+} from cytosol to sarcoplasmic reticulum lumen. Recently, an inherited muscle disorder defined as "congenital pseudomyotonia" (Testoni et al. Vet Rec 163:252, 2008) has been described in Chianina cattle and in a Dutch Improved Red and With cross-breed calf. Cattle pseudomyotonia has been well characterized at both genetic and biochemical levels (Drögemüller et al. Genomics 92:474–477, 2008; Sacchetto et al. Am J Pathol, 174:565–573, 2009; Grunberg et al. Neuromuscul Disord, 20:467–470, 2010). By DNA sequencing of affected calves, we have provided evidence of mutations in ATP2A1 gene. Moreover, our results clearly demonstrated that cattle pathological muscles are characterized by a selective deficiency in Ca^{2+}-ATPase activity. Recently, we have obtained crystals of bovine wild-type SERCA1 protein. On the basis of symptoms and of genetic and biochemical confirmations, cattle pseudomyotonia has been defined as the true counterpart of human Brody disease and bovine species might be used as a suitable non-conventional animal model for investigating the pathogenesis of Brody disease.

C. elegans as a model organism for Limb Girdle Muscular Dystrophy

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Limb Girdle Muscular Dystrophy (LGMD) is a complex disorder affecting muscles around the pelvic and shoulder girdles. There are 12 forms of autosomal recessive LGMD and 7 autosomal dominant LGMD with 15 disease causing genes identified so far. To determine if *C. elegans* can be used as a model organism to study LGMD we have selected 14 *C. elegans* homologues of human LGMD disease causing genes. We have used RNAi feeding vectors and our established transgenic lines and methods to screen these genes for altered muscle protein degradation, synthesis and/or dystrophy. For the genes identified to cause muscular defects we now aim to determine if LGMD drugs can alleviate such defects and further validate *C. elegans* as a suitable model for human LGMD. This work was funded by the UK Medical Research Council (grant G0801271).

Behavioural and functional alterations in a *D. melanogaster* model of Myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is a dominantly inherited disorder affecting skeletal muscles, heart, eyes, endocrine and central nervous system (Harper 2001). The genetic basis consists of an expansion of an unstable (CTG)n triplet repeat on chromosome 19 (Fu et al. 1992; Mahadevan et al. 1992; Aslanidis et al. 1992). Several theories postulate that the expanded CTG repeats disrupt normal cellular processes at RNA, protein or chromatin level (reviewed in Cho and Tapscott 2007). Muscle dysfunction, together with CNS abnormalities, are part of the clinical features of DM1 (Machuca-Tzili et al. 2005). In the past various mechanisms have been suggested to be associated with the muscle and CNS physiopathology of the disease. To further investigate the pathophysiological mechanisms underlying muscular and CNS dysfunctions of DM1 we employed a *D. melanogaster* model using a specific transgenic line containing 60 or more repeated CUG triplets is expressed (Garcia-Lopezet al. 2008). Behavioural and electrophysiological experiments analysed both CNS and muscle function, showing locomotor as well as electrophysiological alterations in DM1 transgenic line. These preliminary data show that *D.melanogaster* is a model to investigate not only the genetic, molecular and structural mechanisms of DM1, but also to exploit CNS and muscle physiopathological alterations of the disease.

Poster abstracts

P.2

The effect of the Glu40Lys mutation of α-tropomyosin on actin-myosin interaction during ATPase cycle

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Mutations in the human TPM1 gene encoding α-tropomyosin expressed in cardiac muscle fibers cause inherited dilated cardiomyopathy, characterized by cardiac dilatation and contractile dysfunction. To understand how the Glu40Lys mutation of α-tropomyosin affects actomyosin interactions, we labeled Cys707 of myosin subfragment-1 and Cys374 of actin with the fluorescent probe 1.5-IAEDANS. These proteins were incorporated into ghost muscle fibres and their conformational states were monitored during the ATPase cycle by measuring polarized fluorescence. The addition of wild-type α-tropomyosin to actin filaments increased the amplitude of the SH1 helix and subdomain-1 movements during the ATPase cycle, indicating the enhancement of the efficiency of work of each cross-bridge. The Glu40Lys mutation inhibited this effect. The Glu40Lys mutation also resulted in the coupling of the weak-binding sub-state of S1 to the strong-binding sub-state of actin thus altering the concerted conformational changes during the ATPase cycle. We suggest that these alterations will result in reduced force production, which is likely to underlie at least in part the contractile deficit observed in human dilated cardiomyopathy. Supported by RFFR (08-04-00960a).

P.3

Cross bridge kinetics after active stretch in skinned fibres

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The steady state force produced by a muscle following active stretch is greater than the corresponding purely isometric force. This phenomenon is called "Residual Force Enhancement" (RFE). Most of the proposed mechanisms of RFE were either related to the development of structural non-uniformities because of sarcomere length instabilities on the descending limb of the force–length relationship, or with the engagement of a passive structural element upon activation that would produce the additional force after stretching. So far there have been hardly any experiments investigating the possibility for a change in cross bridge kinetics after active stretching. The aim of this study was to investigate if RFE was accompanied with changes in cross bridge kinetics. Cross bridge kinetics were evaluated by measuring stiffness in an isometric reference state and in the force enhanced state following active stretch, the time required for cross bridges to redevelop force (Δt) following a quick release, and the rate

of force redevelopment (Ktr) after a quick release. Stiffness and Ktr were not changed after active stretch compared to a purely isometric contraction. Δt after active stretch was smaller than Δt after a purely isometric contraction. Combined, these results suggest that active stretching does not cause an increase in the number of attached cross bridges nor does it affect the rate constants of cross bridge cycling. The strain of a passive element, likely the molecular spring titin, during active stretch and then its recoil when the fibre is released might be responsible for the observed decrease in Δt after stretch.

P.4

Effect of thyroid hormones on actin subdomain-1 movement induced by myosin subfragmant 1 binding in fast and slow rat skeletal muscles

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Orientation and mobility of fluorescent probe *N*-(iodoacetyl)-(1-naphtyl-5-sulpho-ethylenediamine) (1.5-IAEDANS) specifically bound to Cys-374 of actin in ghost muscle fibers isolated from fast and slow glycerinated rat muscles were studied by polarized fluorimetry in the absence and presence of myosin subfragment 1 (S1) in intact rats and in animals with gradual (during 1–5 weeks) reduction of thyroid hormones synthesis (hypothyreosis development) and gradual increase of hormone level (hyperthyreosis development) by injection of 3-iodo-L-thyronin. S1 binding to F-actin of ghost muscle fibers was shown to induce the changes in orientation of the dipoles of fluorescent probes 1.5-IAEDANS and in the relative amount of the randomly oriented fluorophores that indicated the changes in actin subdomain-1 orientation and mobility resulting from the formation of its strong binding with S1. That effect was markedly inhibited by both hypo- and hyperthyreosis development. During the change of thyroid status, the decrease of mobility and the rotation of actin subdomain-1 towards thin filament periphery was found. The maximal effect of hypothyreosis was observed after 34 days of disease and 21 days after hypothyreosis development. It is suggested that the change of thyroid status in muscle inhibits the ability of F-actin to form strong binding with myosin which is essential for force generation.

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P.6

Force-generating capacity of human myosin isoforms extracted from single muscle fibre segments

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Muscle, motor unit and muscle fibre type-specific differences in force-generating capacity have been investigated for many years, but there is still no consensus regarding specific differences between slow- and fast-twitch muscles, which may relate to a number of different confounding factors disguising the function of the molecular motor protein, i.e., the myosin. The aim of this study is to evaluate the force-generating capacity of specific myosin isoforms extracted from human muscle fibre segments in a modified single fibre in vitro motility assay, in which an internal load (α-actinin, actin-binding protein) was added in different concentrations to inhibit force generation of the myosin. After the negative linear relationship between the fraction of moving filaments and the α-actinin concentrations was plotted, both the slope and *x*-axis intercept were used as *force index* to evaluate force production. The force-generating capacity of the β/slow myosin isoform (type I) was weaker ($P < 0.05$) than the fast myosin isoform (type II), but the force-generating capacity of the different fast myosin isoforms types IIa, IIx or IIax were inseparable. In conclusion, (i) a significant difference in force-generating capacity was observed between human slow and fast myosin isoforms; (ii) the modified single fibre in vitro motility assay presents a unique possibility to measure the force-generating capacity of specific myosin isoforms, and also provides a platform for studies on myosin function in the growing disease entity called "myosinopathies" as well as in the different post-translational modifications.

P.8

3-Hydroxy-3-methylglutaryl Coenzyme A reductase inhibitor simvastatin modifies skeletal muscle contraction

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The inhibition and the down-regulation of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR, the key enzyme of the cholesterol biosynthetic pathway) prevents the differentiation of myoblasts into multinucleated syncitia (Martini et al. J Cell Physiol 220:524–530, 2009). Moreover clinical data show that statins, strong HMGR inhibitors used in therapies against hypercholesterolemia, can cause myopathy and occasionally rhabdomyolysis (Dirks and Jones Am J Physiol Cell Physiol 291:C1208–12, 2006). These combined findings bring out the importance of HMGR activity in skeletal muscle physiology. Thus, the purpose of this work was to evaluate whether the inhibition of HMGR activity could cause any putative modifications of muscle contraction. To this aim adult male rats were treated for 3 weeks with a daily intraperitoneal injection of a statin, simvastatin (1.5 mg/kg) and the contractile parameters of extensor digitorum longus (EDL) muscle were evaluated. The obtained data show that the power output and the maximum shortening velocity exerted by EDL of simvastatin treated rats were significantly reduced. Surprisingly, the observed phenomena were not due either to the reduction of the main HMGR end products or to the fiber loss resulting from apoptosis or necrosis but instead to the fiber type IIb-IIa/x shift. These results provide a new mechanism underlying the side effects exerted by simvastatin on skeletal muscle physiology through the contractile parameter worsening.

P.9

Heavy meromyosin head-surface distance on a silanized surface studied using fluorescence interference contrast microscopy

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The in vitro motility assay, where adsorbed myosin motor fragments e.g. heavy meromyosin (HMM) propel actin filaments on nitrocellulose

or silanized surfaces, is frequently used in fundamental studies of actomyosin function. In this connection, understanding of the mode of HMM adsorption can be of importance for the correct interpretation of experimental results. Here, we used fluorescence interference contrast microscopy to measure the distance between the motor domain of HMM and underlying trimethylchlorosilane [TMCS] derivatized SiO_2 surfaces. The actin filaments are held by HMM in rigor 38 ± 2.3 nm (mean ± SEM; estimated from error propagation) above the TMCS surface, suggesting a C-terminal end adsorption of HMM, corresponding to locking of the myosin "hinge" region to the thick filament backbone. The average height of the HMM held actin filaments above the surface did not change appreciably (<±10 nm) upon addition of 1 mM ATP at an ionic strength of ~40 mM. The average height is considerably greater than the distance of 10–15 nm between the thin and thick filaments in the myofilament lattice of skeletal muscle.

P.11

Relative contributions of myosin subfragment-1 and -2 to the compliance of muscle cross-bridges: X-ray diffraction evidence

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In the X-ray diffraction pattern from skeletal muscle the 3rd order myosin-based meridional M3 reflection originates from the axial repeat of myosin cross-bridges along the thick filament. Changes in the intensity (I_{M3}), spacing (S_{M3}), and fine structure (R_{M3}) of the M3 reflection in contracting muscle have been measured in many different protocols (Linari et al. PNAS 97:7226, 2000; Piazzesi et al. Nature 415:659, 2002; Reconditi et al. Nature 428:578, 2004; Huxley et al. J Mol Biol 363:743, 2006). The results are explained with model simulations based on (1) the crystallographic structure of the myosin head (subfragment-1, S1), integrated with the tilting lever arm hypothesis (Rayment et al. Science 261:50, 1993), (2) the presence of a fixed periodic mass attributed to detached myosin heads and (3) the assumption that all the compliance of the cross-bridges resides in S1. (Seebohm et al. Biophys J 97:806, 2009). Here the possibility that a substantial proportion of the cross-bridge compliance is provided by the subfragment-2 (S2) link between the head and the thick filament (Knupp et al. J Mol Biol 390:168, 2009) is analysed by adding a variable compliance in S2 and testing the resulting model in the different protocols mentioned above and, moreover, against *I*M3 changes induced by rapid length changes imposed on the muscle fibre in rigor (Dobbie et al. Nature 396:383, 1998). The results show that S2 does not significantly contribute to the cross-bridge compliance.

P.12

Strain response of the myosin essential light chain in skeletal muscle fibres detected by FLIM

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We applied fluorescence lifetime imaging microscopy (FLIM) to map the microenvironment of the myosin essential light chain (ELC) in skeletal muscle fibres. Four ELC mutants containing single cysteine residue at different positions in the C-terminal half were labeled with 7-diethylamino-3-((((2-iodoacetamido)ethyl)amino)carbonyl)coumarin and introduced into permeabilized psoas muscle fibres under conditions that favour exchange with the native light chain. The fibres were examined under Leica SP5 microscope equipped with a time-correlated single photon counting module. The fluorescence decay in each pixel of FLIM images was fitted with a single exponential and the mean fluorescence lifetime in the A-band regions was found. The mean lifetimes in relaxed fibres were 1.44, 1.64, 1.73 and 1.83 ns, for ELC-142, ELC-127, ELC-160 and ELC-180 respectively. When in rigor, lifetime increased significantly for all label positions, which may be related to a change in conformation of ELC with respect to the heavy chain. However, when 1% stretch was applied to the rigor fibres, the lifetime of ELC-127 and ELC-180 decreased, but did not change in cases of ELC-142 and ELC-160, where the labels are located at the opposite ends of helix F. The decrease in lifetimes of ELC-127 and ELC-180 likely results from the interactions between two halves of ELC, and between the C-terminal area of ELC and the N-terminal subdomain of the heavy chain, respectively. Supported by BBSRC.

P.13

Insulin mediates rapid titin phosphorylation via NO-dependent activation of PKG

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The passive properties of the giant protein titin (>3 MDa) are defined by the expression ratio of the two cardiac isoforms N2BA and N2B in the sarcomere, and can be dynamically modulated via phosphorylation, e.g. by cAMP or cGMP-dependent protein kinases (PKA, PKG). In cultured embryonic rat cardiomyocytes (ECM) titin phosphorylation is significantly increased upon insulin treatment. Since insulin is known to activate PKG in an NO-dependent manner, we tested whether PKG is responsible for the insulin-induced changes in titin phosphorylation. Cultured embryonic rat cardiomyocytes (ECMs) were treated with insulin (175 nM) for 5–60 min in the absence or presence of specific inhibitors of the NO-dependent PKG-pathway. Titin phosphorylation was analyzed in 2% SDS PAGE using the phosphoprotein stain ProQ-Diamond. Activation of PKG was confirmed by detecting phosphorylation of the target protein vasodilator-stimulated phosphoprotein (VASP). Titin phosphorylation reached a maximum after 15 min of insulin treatment and was increased 1.9-fold for N2BA-titin and 1.6-fold for N2B-titin compared to untreated ECMs. The insulin-induced effect on titin phosphorylation was prevented by inhibition of nitric oxide synthase (L-NAME 100 μM), guanylate cyclase (ODQ 10 μM) or PKG (Rp-8-PET-Br-cGMP 100 nM). We therefore conclude that insulin modulates PKG-dependent titin phosphorylation by activating the NOS/PKG pathway, and thereby may modify titin-based passive stiffness.

P.14

Structural and folding studies on the titin A-band/M-band transition region

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The giant muscle protein titin is the largest protein known to date. It spans over the length of a half sarcomere from Z-disc to the M-band and shows important functions in elasticity, contraction and assembly of the sarcomere. Next to Ig-like and fibronectin type 3 (FnIII)

domains, of which it is mainly composed, it possesses a single catalytic domain (titin kinase), which is regulated by an unknown mechanism and located at the transition region between A-band and M-band. Although this region is of special interest due to the presence of titin kinase, only little is known about its structure and organization.

Our work is focused on the structural and kinetic features of the aforementioned region. The multi-domain organization of the titin A-band/M-band transition region was characterized by complementary methods. We solved by X-ray crystallography the Ig-like tandem A164-A165 and performed SAXS measurements on different titin fragments, encompassing the domains A164 to M2. Our data show that this region is divided into a rigid and a flexible part located N-terminally and C-terminally of the kinase domain respectively.

In order to complement these structural findings, unfolding studies on the tandem A164-165 were performed. A164-A165 unfolds like a cooperative unit and its unfolding rate is significantly lower than those of the individual domains. This implies that the unfolding of A164-A165 is not favored and the tandem is very likely force-resistant.

P.15

Cyclic GMP enhancing therapy promotes titin phosphorylation and corrects high cardiomyocyte passive stiffness in diastolic heart failure

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Myofiber passive stiffness is lowered by phosphorylation of the giant sarcomeric protein titin, with beneficial effects on diastolic function. Titin can be phosphorylated by cGMP-activated protein kinase (PK)G, a pathway stimulated by B-type natriuretic peptide (BNP) or PDE5A inhibitor (sildenafil). Whether titin phosphorylation and stiffness are affected by PKG activation in vivo had not been studied. Here we examined how dogs with experimental hypertension and diastolic dysfunction induced by renal wrapping respond to treatment with beta-blockers, sildenafil, and BNP, in terms of altered titin phosphorylation and passive stiffness.

Phosphorylation of titin isoforms was studied by gel electrophoresis using SYPRO Ruby (total protein) and ProQ Diamond (phospho protein) stain and was reported as ratio of phosphorylated titin isoforms (P-N2BA/P-N2B) and as total titin phosphorylation. Isolated permeabilized myocytes from left ventricular (LV) tissue samples of healthy control ($n = 8$) and diastolic heart failure (DHF) dogs ($n = 8$), as well as LV biopsies of DHF dogs treated with beta-blocker (DHF + β; $n = 4$), followed by sildenafil ($n = 4$) and BNP ($n = 4$) were attached to a force transducer and passive tension (Fpassive) was measured between 1.8 and 2.4 μm sarcomere length (SL).

The P-N2BA/P-N2B ratio was low in controls, increased in DHF, remained high after beta-blockers, but significantly decreased with sildenafil and BNP. Total titin phosphorylation was low in DHF and DHF + β, significantly increased in sildenafil, and remained high with BNP. Fpassive at 2.2 μm SL was low in control myocytes, elevated in DHF and DHF + β, but returned to control levels with sildenafil and remained low with BNP.

Acute cGMP enhancing therapy with sildenafil and BNP improves LV diastolic function through correction of a titin phosphorylation deficit, particularly of the stiff N2B titin isoform, thereby reducing myocyte passive stiffness.

P.16

Innervation-dependent distribution of myosin VI in human and rat skeletal muscles

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Myosin VI (MVI), a reverse-direction motor protein moving towards the minus-end of actin filaments, is engaged in a variety of cellular processes such asactin cytoskeleton dynamics and organelle trafficking, maintenance of Golgi morphology and protein secretion, endocytosis, cell migration and possibly in gene transcription. It was shown that a point mutation (H236R) in the MVI gene leads to deafness and mild symptoms of hypertrophic cardiomyopathy. We have shown that myosin VI is expressed in several types of human and rat skeletal muscles. Studies on denervated muscles, obtained from rats either with a sciatic nerve cut or from *amyotrophic lateral sclerosis* (ALS) model animals, and from human muscle biopsies from patients diagnosed with neurogenic muscle disorders, revealed that the amount of the protein depended on muscle innervation, and was significantly increased in the diseased tissues. Moreover, myosin VI was present in the postsynaptic region of the neuromuscular junction and this localization was abolished in the denervated muscles, thus implying an influence of neuronal factors determining distribution of MVI in the muscle. Our studies indicate that myosin VI may be involved in the neuron-muscle transmission in the innervation-dependent manner.

P.17

Effects of short-term hypogravity on M-line transversal stiffness and one possible way to interpret stiffness data

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The search of mechanosensors is an actual problem because the rearrangement of mechanical stress inside the fiber could be direct signal for some adaptive cell programs. For the muscle fiber, one of the probable sensors of such kind could be located in M-line (A.A. Shabarchin, A.K. Tsaturyan 2010). Thus, we evaluated changes in condition of M-line by means of atomic force microscopy (AFM). As a model of hypogravity, we used rodent antiorthostatic hindlimb unloading of 3-day duration.

The data on stiffness during different activation conditions were the following: significant decrease in experimental series (from 3.69 to 3 pN/nm in relaxed state, from 7.9 to 4.1 pN/nm in activated state and from 10.3 to 7.27 pN/nm in rigor). Therefore, the relative changes of stiffness in every series vary significantly that could be the evidence of different dependence of stiffness from activation. To analyze the impact of protein structures to M-line stiffness, we designed the model of interaction of AFM probe with M-line with

elastic plate theory. We bring some constants, which are the coefficients of approximation function. Each of these constants has its own physical meaning connected with concentration of protein levels and probably their condition.

Therefore, the observed changes in this constants could be then be interpreted as evidence of probable changes in particular proteins level (determined by their assumed role in mathematical model).

P.18

AHNAK1 and AHNAK2 are costameric proteins: AHNAK1 affects transverse skeletal muscle fiber stiffness

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We used specific AHNAK1 and AHNAK2 antibodies to analyzed the detailed localization of both proteins in mouse single skeletal muscle fibers isolated from flexor digitorum brevis (FDB) of 12 week old male mice. Co-localization of AHNAK1 and AHNAK2 with vinculin clearly demonstrates that both proteins are components of the costameric network. No AHNAK expression was detected in the T-tubule system. To induce damage to the muscle fibers, a 5 × 5 μm area of the fibers were irradiated with a 30 mW argon-laser) using a Zeiss-LSM 510 META confocal microscope in Tyrode solution with 2.5 μM FM1-43F. After membrane damage, we analyzed fluorescent dye entry into AHNAK1$^{-/-}$-and wt fibers. In the presence of Ca^{2+}, both fiber types showed a slow dye inflow due to rapid membrane resealing. Membrane resealing kinetics of mouse AHNAK1$^{-/-}$ FDB fibers were not significantly different from wt fibers. Atomic force spectroscopy measurements (AFM) were performed using CellHesion 200 (JPK Instruments, Germany) mounted on an inverted optical microscope (Axio Observer D1, Carl Zeiss). Silicon tipless cantilevers (Arrow TL1, NanoWorld) with a nominal spring constant of 0.03 N/m were modified with 11 μm-glass spheres. The cantilever sensitivity and spring constant were determined using the in-built calibration routine (thermal noise method). The Young's modulus was determined for each force indentation curve by fitting the extend curves using the Hertzmodel for spherical indenters. For an indentation of 50 nm, we calculate an Elastic modulus (E) of about 700 Pa for AHNAK1 deficient fibers, which is significantly ($P < 0.001$) higher compared to the Elastic modulus of about 350 Pa for wt fibers.

P.19

Desmin content in the rat and Mongolian gerbils limb muscles at different stages of gravitational unloading

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Result of experiments, which were held after space flight of FOTON-M3 (2007, Russia), showed that atrophic changes in Mongolian gerbil's postural muscles are not so dramatic such as in the rats after analogical microgravity influence (Lipets E.N. et al. 2008).

We compared desmin content by means of immunoblotting in m. soleus (Sol), m. medial gastrocnemius (MG), m. tibialis anterior (TA) of rat and gerbil under 1, 3, 7, 12-days of gravitational unloading. Bioethics committee of SSC RF IBMP RAS approved all animals' procedures.

Desmin content in rat Sol significantly decreased on 25% and recovered to control after 12 days. At the same time desmin content in gerbil's Sol reduced on 30% after 1 day of disuse and restored up to control level after 7 days. In MG and TA in both rat and gerbil, desmin content increased 1.5 times after 1 in rat and 3 days in gerbil and decreased to control level after 12 days.

Such dynamics of desmin content in Sol could be connect with prevailing calcium-dependent proteolysis, in MG and TA there could, probably, be the opposite effect caused by increased EMG-activity. Differences in desmin dynamics between rat and gerbil can be explained by inherent features of this species, for example by water-salt balance. This work was supported by RFBR grant 10-04-00106-a.

P.20

Unravelling the 3D structure of vertebrate muscle M-band

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The sarcomeric M-band in vertebrate muscle maintains the uniform hexagonal lattice of the thick filaments in the middle of the A-band by crosslinking the neighbouring filaments. The crosslinks have different appearances in different muscle types. In longitudinal sections, the M-band in fast muscle comprises 3–5 distinct lines of separation ~22 nm labelled M6′, M4′, M1 (central), M4 and M6. In transverse sections the M-band has a distinct hexagonal network. The M-band in slow muscle comprises either a 2-line or a "fuzzy" M-band appearance longitudinally, and a less distinct network in transverse sections. The M-band consists of several proteins, including myomesin, M-protein and titin. Structural organization of the M-band has not been rigorously investigated. We have carried out electron tomography of the M-band in fish fast muscle in which the thick filaments are organised in a simple lattice, followed by meticulous sub-volume averaging. High-resolution data of the M4′ to M4 region show a set of 3 and 2 nodes interconnecting the thick filaments. We have also derived new information on the backbone structure of the thick filaments over the whole M-band and bare-region (M-region). We show that the backbone structure consists of 3 closely-packed sub-filaments from M6′ to M4′ and M4 to M6. These 3 sub-filaments separate and rotate axially from M4′ to M4. We present new models of the M-band and the thick filament packing in the M-region.

P.21

Regulation of *Drosophila* myosin-7a by a protein binding partner

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The MgATPase of myosin-7a (M7a) from Drosophila requires large amounts of actin to obtain Vmax (KATPase = 40 μM). EM reveals that M7a is single-headed and the tail bends back upon the head. C-terminal truncations that remove as little as 99 residues behave are activated to the same Vmax as with M7a, but have KATPase of 1 μM. These mutations do not fold suggesting that the folding is the basis for weak apparent affinity for actin. A yeast 2-hybrid screen using the last FERM domain uncovered a binding partner we termed DMAP. DMAP binds M7a and shifts the high KATPase values to much lower

levels (2 μM). We used Drosophila S2 cells as a model for studying the localization and function of these proteins. GFP-M7a or mCherry-DMAP expressed alone in S2 cells are diffuse with no actin co-localization. In both cases the cells remain rounded with smooth edges. However co-expression of GFP-M7a and mCherry-DMAP results in a marked shape change with large areas of ruffling membrane and numerous filopodia. The two proteins are extensively co-localized and are found predominantly in actin-rich regions and in filopodia. There is a tendency for the two proteins to be localized near the tips of filopodia. Hemocytes are phagocytic cells found in the hemolymph of larvae. Hemocytes from larvae challenged with bacteria prior to being collected show numerous filopodia, in contrast to hemocytes isolated from unchallenged larvae which are rounded. M7a and DMAP are both present in the filopodia of activated hemocytes.

P.22

Ahnak1 modulates L-type Ca^{2+} channel inactivation of rodent cardiomyocytes

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Ahnak1, a giant 700-kDa protein, has been implicated in Ca^{2+} signalling in various cells. Previous work suggested that the interaction between ahnak1 and Cavβ_2 subunit plays a role in L-type Ca^{2+} current (I_{CaL}) regulation. Here, we performed structure–function studies with the most C-terminal domain of ahnak1 (188 amino acids) containing a PxxP consensus motif (designated as 188-PSTP) using ventricular cardiomyocytes isolated from rats, wild-type mice (WT), and ahnak1-deficient mice. In vitro binding studies revealed that 188-PSTP conferred high affinity binding to Cavβ_2 (K_d ~60 nM). Replacement of proline residues by alanines (188-ASTA) decreased Cavβ_2 affinity about 20-fold. Both 188-PSTP and 188-ASTA were functional in ahnak1-expressing rat and mouse cardiomyocytes during whole cell patch-clamp. Upon intracellular application they increased the net Ca^{2+} influx by enhancing I_{CaL} density and/or increasing I_{CaL} inactivation time course without altering voltage-dependency. Specifically 188-ASTA, which failed to affect I_{CaL} density, markedly slowed I_{CaL} inactivation resulting in a 50–70% increase in transported Ca^{2+} during a 0-mV depolarising pulse. Both ahnak1 fragments also slowed current inactivation with Ba^{2+} as charge carrier. By contrast, neither 188-PSTP nor 188-ASTA affected any I_{CaL} characteristics in ahnak1-deficient mouse cardiomyocytes. Our results indicate that the presence of endogenous ahnak1 is required for tuning the voltage-dependent component of I_{CaL} inactivation by ahnak1 fragments. We suggest that ahnak1 modulates the accessibility of molecular determinants in Cavβ_2 and/or scaffolds selectively different β-subunit isoforms in the heart.

P.23

PKA phosphorylation of L-type calcium channel β_2 subunit (Cavβ_2) alters its interaction with ahnak1

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Ahnak1, a giant plasma membrane support protein interacts with the intracellular Cavβ_2 subunit of the cardiac L-type calcium channel and is involved in L-type calcium current (I_{CaI}) regulation. Previous studies suggested that PKA phosphorylation of Cavβ_2 on Ser478 and Ser479 is necessary to modulate I_{CaI}. But PKA regulation of I_{CaI} could not be observed upon heterologous expression of the channel subunits in Xenopus oocytes (which lacks ahnak1). We identified novel PKA phosphorylation sites in Cavβ_2 on S295 and S575. To study the potential impact of PKA phosphorylation on ahnak1/Cavβ_2 and ahnak1/Cavα1CloopI-II interaction, we generated two Cavβ_2 mutants. First S295 was replaced by alanine to prevent phosphorylation and second, replaced by glutamic acid to mimic phosphorylation. The interactions of Cavβ_2 subunit variants with Cavα1CloopI-II and ahnak15462-5535 were analyzed using Surface Plasmon Resonance technology. We found that phosphorylation of Cavβ_2 did not modify the interaction with Cavα1CloopI-II, but the Cavβ_2E295 variant and PKA phosphorylation of S295, S478, S479 and S575 of Cavβ_2 significantly decreased the binding affinity to ahnak15462-5535. These results show that the additional Cavβ_2 phosphorylation site S295 is functional and therefore may play a role in calcium channel regulation by interacting with ahnak1. Phosphorylation-deficient Cavβ_2 mutants and ahnak1 are currently expressed in Xenopus oocytes and PKA regulation of I_{CaI} is monitored using Two- Electrode Voltage Clamp.

P.25

Essential role of TRPV2 ion channels in the sensitivity of dystrophic muscles to eccentric contractions

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Duchenne myopathy is a lethal disease due to the absence of dystrophin, a cytoskeletal protein associated to the plasma membrane. The lack of dystrophin causes a severe and progressive muscle degeneration, resulting in muscle atrophy and fibrosis. In a normal muscle fibre, dystrophin is associated through its carboxy-terminal end, to a complex of proteins and glycoproteins anchored in the plasma membrane (Dystrophin-associated-Proteins; DAP), containing, in particular, sarcoglycans, dystroglycans and syntrophins. Through its amino-terminal end, dystrophin binds to cytoskeletal actin. The lack of dystrophin induces a reduction of expression of DAPs and promotes a disruption of the physical link between the cytoskeleton and the extracellular matrix. The consecutive disorganisation of the cytoskeleton leads to a disregulation of several ion channel types. We previously showed the disregulation of store-dependent and/or mechanosensitive channels of the TRP channels family (Vandebrouck et al. J Cell Biol, 158, 1089–1096, 2002) in *mdx* muscle.

Fast-twitch muscles from dystrophin-deficient mice (*mdx*) typically present an exaggerated susceptibility to eccentric work characterized by an important loss of isometric force and an increased membrane permeability after repeated lengthening contractions. We investigated the possible involvement of TRPV2, a mechanosensitive channel expressed in muscle, in this process and show that *mdx* muscles are largely protected from eccentric work-induced damage when they overexpress a dominant negative mutant of TRPV2 ion channel (non permeant mutant of TRPV2 isoform, see Iwata et al. Hum Mol Genet 18, 824–834, 2009).

This observation points out the role of TRPV2 channel in the physiopathology of Duchenne muscular dystrophy.

P.26

Lack of calsequestrin in the SR lumen causes free [Ca^{2+}] fluctuation and SR depletion during repetitive stimulation

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The sudden increase in cytosolic Ca^{2+}-elicited during muscular contraction (also known as Ca^{2+}-transients) have been well characterized, but little is known about the free [Ca^{2+}] dynamics inside the sarcoplasmic reticulum (SR). A genetically-targeted FRET-based Ca^{2+} indicator (D1ER) allowed us to investigate SR Ca^{2+}-handling with high time resolution (9 ms or less). The impact of calsequestrin (CASQ) on intraluminal SR [Ca^{2+}] was studied in enzymatically dissociated FDB muscle fibres from wild type (WT) and CASQ knockout mice, lacking either isoform 1 (CASQ1-null) or both isoforms (dCASQ-null) 7 days after transfection with the cDNA coding for D1ER. SR free [Ca^{2+}] measurements were performed at rest and during repetitive stimulation at 1, 5, 20 and 60 Hz. The SR free [Ca^{2+}] at rest did not differ between WT ($n = 26$), CASQ1-null ($n = 25$) and dCASQ-null ($n = 21$) fibres. During electrical stimulation on the other hand, while the changes were rather small in WT, in CASQ1 and dCASQ-null fibres much greater reductions in SR [Ca^{2+}] were recorded. These intraluminal Ca^{2+}-fluctuations were progressively larger with increasing stimulation frequency. At high stimulation frequencies (60 Hz) the SR of CASQ1 and dCASQ-null fibers became virtually depleted of Ca^{2+} during a short tetanus (0.5 s). We also analyzed Ca^{2+} reuptake during and after the trains of stimuli: data collected indicates that re-uptake is governed by 3 temporally distinct processes with rate constants of 50, 1–5 and 0.3 s^{-1} (at 26°C). Interestingly, Ca^{2+}-transients, detected with Fura-2, were characterize by a marked decline during repetitive stimulation. This indicates that the SR calcium released is insufficient for sustained contractile activity in fibres lacking CASQ. In conclusion, our results underline the essential role of CASQ as intra SR Ca^{2+} buffer and show that in the absence of CASQ the SR content is greatly reduced.

P.27

How does coordination between parvalbumin and mitochondria regulate the Ca^{2+} homeostasis in muscle?

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Parvalbumin (PV) is a Ca^{2+}-binding protein highly expressed in fast-twitch muscles and GABAergic neurons and is considered to principally function as an intracellular modulator of Ca^{2+} signals. An inverse relationship exists between PV and mitochondria volume: ectopic PV expression in neurons decreases mitochondrial volume, while increasing it in Purkinje cells and fast-twitch muscles of $PV^{-/-}$ mice. Strikingly, the $PV^{-/-}$ fast-twitch muscle phenotype is unchanged with respect to contractile elements. We investigated the PV/mitochondria antagonism in muscle tissue with respect to the Ca^{2+}-dependent processes and signaling pathways leading to either muscle fiber-type specific protein expression and/or pathways involved in mitochondria biogenesis. We performed experiments in freshly isolated PV-/- and WT muscles and in C2C12 muscle cells; in PV-ir negative control cells and stably PV-transfected clones. Both clone types were subjected to treatments affecting Ca^{2+} homeostasis. Since mitochondria also act as transient Ca^{2+} stores/buffers in muscle cells, the mitochondrial volume/mass was investigated by 3D reconstruction and flow cytometry, respectively, under control conditions and after altering intracellular Ca^{2+}. In C2C12 and isolated muscle fibers levels, factors linked to mitochondria biogenesis or implicated in Ca^{2+} signaling were analyzed at the mRNA and/or protein level. Finally, we used imaging techniques to investigate the Ca^{2+} dynamics and PV's role in Ca^{2+}-buffering.

P.28

Loss of the Ank1.5 muscle specific isoform causes morphological alterations and contractile response impairment in adult mouse skeletal muscles

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The small muscle specific ankyrin 1.5 (ank1.5), a muscle-specific splice variant of the Ank1 gene, has been shown to interact with the myofibrillar protein obscurin, providing the first evidence for a link between the sarcoplasmic reticulum (SR) and the contractile apparatus in striated muscles. To further understand the role of ank1.5 protein, we developed a mouse strain KO for ank1.5. The ank1.5 KO mice are viable and when young they do not present evident alterations in skeletal muscles. However, in old ank1.5 KO mice we observed areas of excessive contracture that were more evident in the diaphragm. Electron Microscopy revealed areas of myofilament degeneration that alternate with apparently normal regions. Interestingly, both light and Electron Microscope revealed the development of tubular aggregates in fast fibers of old ank1.5 KO mice. Accordingly, we also found that the contractile performance of diaphragm was weaker in 1-year old ank1.5 KO compared to age matched control mice. Namely, tetanic force was reduced and half relaxation time was increased. Evans Blue Dye test revealed some positive fibers, indicating cell damage in ank1.5 KO again in diaphragm. Moreover, resistance to fatigue of ank1.5 KO animals as from the treadmill endurance test was reduced. Altogether these data prove that ablation of ank1.5 results in damage to the contractile apparatus, tubular aggregates development and in the worsening of the contractile function in aging skeletal muscle.

P.29

The effect of eccentric contractions on sarcoplasmic reticulum Ca^{2+} handling in fast-twitch skeletal muscle of the rat

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The aim of this study was to investigate the effects of eccentric contractions (ECC) on the sarcoplasmic reticulum (SR) Ca^{2+} handling and calpain activity in skeletal muscle. The left anterior crural muscles of male Wistar rats were exposed to 200-repeated ECC.

The contralateral muscles were used as control. Immediately after 3 and 6 days following ECC, the extensor digitorum longus and tibialis anterior muscles were removed and used for measures of force output and for biochemical analyses, respectively. Immediately after ECC, isometric tetanic force at 20 Hz was decreased by 80.7% and depressed force output did not revert to pre-exercise levels during 6 days of recovery. SR Ca^{2+} release rate was not altered immediately after ECC but decreased beyond 3 days. This alteration was accompanied by a reduction in the protein content of ryanodine receptor (RyR) and an elevation of calpain activity. In contrast to SR Ca^{2+} release, no change was found in SR Ca^{2+} uptake rate. These results suggest that disturbances in SR Ca^{2+} release function may account, at least in part, for ECC-induced reductions in force and power which can take a number of days to recover and that the inability of SR to release Ca^{2+} would result from RyR degradation.

P.30

Time course of changes in vitro sarcoplasmic reticulum Ca^{2+} handling and sarcolemmal Na^+-K^+-ATPase activity during eccentric contractions

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In this study, we examined whether eccentric contraction (ECC) would cause alterations in sarcoplasmic reticulum (SR) Ca^{2+} handling and sarcolemma Na^+-K^+-cycling properties as assessed in vitro. For this purpose, rat extensor digitorum longus and tibialis anterior were exposed to repeated (up to 500 cycles) ECC or isometric contraction (ISC) in situ and used for measures of force output and for biochemical analyses, respectively. Isometric maximal tetanic force, SR Ca^{2+}-ATPase activity, SR Ca^{2+}-uptake and release rate and Na^+-K^+-ATPase activity were measured in vitro. Contraction-induced decreases in maximal tetanic force were greater in ECC-treated muscle than in ISC-treated muscle. Intactness of SR membrane as assessed with the use of Ca^{2+} ionophore and SR Ca^{2+}-uptake and release rate were unchanged either in ECC- and ISC-treated muscles. Whereas depressions in SR Ca^{2+}-ATPase activity were observed both in ECC- and ISC-treated muscles, significant depressions in Na^+-K^+-ATPase activity occured only in ECC-treated muscle. These results suggest that force developed by ECC-contracted muscle may be affected to a greater extent by changes in the excitability of sarcolemma compared with ISC-contracted muscle. Future studies are needed to establish the mechanism underlying ECC-induced dysfunction of SR Ca^{2+}-ATPase and Na^+-K^+-ATPase.

P.31

Calsequestrin-1 is involved in calcium transient amplitude induced by electrical stimulation of fast-twitch muscle fibres

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Amplitude of calcium (Ca^{2+}) transients and structure of Ca^{2+} release units (CRUs) are significantly altered in Calsequestrin1 (CS1)-null mice (Paolini et al. 2007). In this work, the impact of CS1 ablation was characterized by recording Fura2 loaded CS1-null FDB fibers stimulated with low (1 Hz) and high (60 Hz) trains of pulses. Beside a significant decrease of peak height upon low frequency stimulation, variable time-dependent decline of the Fura-2 signal during high frequency stimulation was observed in comparison to WT. In order to study if CS1 was involved, exogenous mouse CS1 was expressed in adult CS1-null FDBs by in vivo DNA electrotransfer to obtain functional reversion of the phenotype. Exogenous CS1 was correctly targeted to CRUs and positioned at the junctional Sarcoplasmic Reticulum, in close proximity of Ca^{2+} release sites. At proteomic level CS2, Sarcalumenin, Triadin and Junctin did not significantly change upon CS1 expression. Successfully, transient peak height in low frequency stimulation showed a significant increase upon CS1 expression, moreover the decline of Fura2 fluorescence signals during high frequency (60 Hz) stimulation was abolished. The present results provide strong evidence that CS1 contributes to the amount of released Ca^{2+} elicited with low frequency stimulation and is a key reservoir of mobilizable Ca^{2+} to support high frequency stimulation in fast-twitch fibres.

P.32

Glycogen modulates EC coupling in elite triathletes, by affecting SR Ca^{2+} release rate

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Little is known about the precise mechanism that relates skeletal muscle glycogen to muscle fatigue. However, based on data from in vitro studies I has been proposed that glycogen affects sarcoplasmic reticulum (SR) function. The aim of the present study was to examine the effect of glycogen on SR function in elite triathletes ($n = 15$, VO_2max 70 ± 1 ml kg^{-1} min^{-1}) before, immediately after, and 4 and 22 h after a fatiguing 4 h cycling at 75% HRmax. Triathletes were randomised to receive either water or carbohydrate (CHO) during the first 4 h recovery, and thereafter all received CHO enriched food. The muscle glycogen was reduced to 33 ± 4% following exercise (708 ± 28 to 239 ± 29 mmol kg^{-1} dw), while SR Ca^{2+} release rate was reduced to 84 ± 3% of initial. During the 4 h recovery with CHO, the SR Ca^{2+} release rate was fully normalized and glycogen was noticeably recovered (59 ± 2% initial). However, in the absence of CHO during the first 4 h recovery, the muscle glycogen and SR Ca^{2+} release rate remained low and reduced (39 ± 4 and 83 ± 3%, respectively), with both parameters being normalized after the remaining 20 h recovery with CHO. Interestingly, the SR Ca^{2+} release rates and muscle glycogen contents was highly significant correlated ($P < 0.0001$, $r = 0.57$). There was no effect on SR Ca^{2+} uptake. These observations from trained humans, is in agreement with the emerging concept of glycogen having a regulatory effect on the SR release function.

P.33

Initial characterization of CASQ1/CASQ2 knockout (double CASQ-null) mice

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Calsequestrin (CASQ), the major Ca^{2+}-binding protein of the sarcoplasmic reticulum (SR), is expressed in two different isoforms in adult skeletal muscle: CASQ1 and CASQ2, which is co-expressed with CASQ1 only in slow-twitch fibers. CASQ1-null mice, in which CASQ2 is still expressed in about 50% of Soleus fibers, show: (a) structural and functional alterations of the EC coupling machinery, which are more pronounced in EDL than in Soleus; (b) higher rate of spontaneous mortality of male animals. Here we present the initial characterization of mice lacking both CASQ isoforms (double(d)CASQ-null), as confirmed by western blot analysis. dCASQ-null mice are viable and breed normally, however they present a rate of spontaneous mortality of male animals even higher than that of CASQ1-null animals, possibly due to additional cardiac problems. Whereas the overall phenotype of these mice is similar to that of CASQ1- null mice, ablation of CASQ2 resulted in worsening of the Soleus phenotype as many fibers show severe structural damage. The contractile response of dCASQ-null Soleus muscle, however, is only moderately impaired, with alterations in time and force parameters not significantly different from those observed in CASQ1-null mice. These findings suggest that, while the lack of both CASQ1 and 2 exacerbates the structural phenotype of muscles rich in slow twitch fibers, the contractile response is less dependent on CASQ in slow than in fast muscles.

P.34

Progressive un-coupling of mitochondria from calcium release sites in ageing: implications for muscle performance

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An impairment of the EC coupling mechanism and a miss-function of mitochondria have been both proposed to contribute to the age-related decline of muscle performance that accompanies aging. EC coupling in muscle fibers occurs at specialized intracellular junctions called calcium release units (CRUs), or triads, which are structurally and functionally connected to mitochondria (Boncompagni et al. 2009). Here we have studied the morphology, frequency, sarcomeric-localization, and coupling of CRUs/mitochondria in EDL from ageing male WT mice using electron microscopy. Our results indicates that the number of CRUs/$100\,\mu m^2$ of longitudinal section in aging mice ($n = 4$, 25–35 months) decreases compared to adult mice ($n = 5$, 3–12 months): 93 ± 9 vs. 79 ± 8. In addition, also the number of mitochondria-profiles/$100\,\mu m^2$ decrease with age: 54 ± 7 vs. 43 ± 6 respectively. In addition, we have assessed the positioning of mitochondria in respect to myofibrils and triads: a) the number of mitochondria at the A band (misplaced) slightly increases with age (9.3 vs. 2.5%). These changes cause a significant decrease in the number of CRUs-mitochondria couples: 39 ± 5 vs. 26 ± 5. Our results indicates an age-related spatial re-organization of the EC coupling and mitochondrial apparatuses, which causes a significant decrease in the number of CRUs/mitochondria functional couples. These structural changes may contribute to the decrease of specific force and endurance of skeletal muscle associated to ageing.

P.35

Molecular determinants of association of triadin to the junctional sarcoplasmic reticulum of skeletal muscle cells

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The sarcoplasmic reticulum (SR) of skeletal muscle cells is a continuous network of membranes in which some specific domains (i.e. the longitudinal SR and the junctional SR) can be clearly distinguished. Although each of these domains is characterized by a specific protein composition, the mechanisms leading to protein targeting to these domains are still unknown. In particular, the junctional sarcoplasmic reticulum (jSR) of skeletal muscle cells contains several proteins that participate to the mechanisms of Ca^{2+} release in the process of excitation–contraction coupling. Among these proteins, ryanodine receptor, triadin, junctin and calsequestrin have been found to associate into a stable complex. We recently reported that assembly of jSR domains is accompanied by a strong decrease in the mobility fraction of jSR proteins (Cusimano et al. PNAS 2009). In particular, we found that the mobility of triadin appeared to be mediated by its intraluminal region (aa 232–729). In order to identify the minimal regions required for association of triadin to the jSR, deletion mutants of the luminal domain (triadin 232–440 and triadin Δ441–729) were generated and expressed in primary muscle myotubes. Analysis of the mobility fraction of these mutants showed that they do not differ from wild type triadin, indicating that either one of the two regions is sufficient to provide a strong association of the protein to the jSR. Interestingly, we found that the luminal region of triadin contains several defined domains, including a coiled coil region and short amino acids repeats, that are present in the region between aa 232–440 and aa 441–729, where they may mediate protein–protein interactions. Results on the role of these amino acid repeats and domains in mediating triadin association with the jSR will be reported.

P.36

Detection of elementary calcium release events in skeletal muscle by stationary wavelet method

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The elementary events of intracellular calcium release in muscle cells (sparks and embers) are studied frequently by confocal microscopy. The large quantity of images and large number of events require automatic detection procedures based on signal processing methods. First methods were based on thresholding procedures, but recently wavelet transforms were also introduced. We have implemented a set of algorithms based on one and two dimensional versions of the à trous wavelet transform. Algorithms were used to perform spike filtering, denoising and detection procedures. We studied in detail the effect of user adjustable parameters on the efficiency of the algorithm. Methods were given to avoid false positive detections which are the consequence of the background noise. In order to establish the efficiency and reliability of the algorithms, various tests were performed on artificial and experimental images. Spark parameters were compared calculated using the traditional and the wavelet methods. We found that the latter method is capable of identifying more events with better accuracy on experimental images. Furthermore, we extended the wavelet based transform from sparks to long-lasting small-amplitude events as embers. The method not only solved their automatic detection but enabled the identification of events with small amplitude that otherwise escaped the eye, rendering the determination of their characteristic parameters more accurate. Supported by OTKA K75604, NK78398.

P.37

Unfolded protein response is activated in heart carrying the mutation R33Q of calsequestrin (CS2) linked to catecholaminergic polymorphic ventricular tachycardia

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Isoform 2 of Calsequestrin (CS2), is the main calcium-binding protein of sarcoplasmic reticulum (SR), expressed both in cardiac and in skeletal muscles. CS2 not only acts as an SR Ca^{2+} sensor, but also regulates SR Ca^{2+} release via interactions with triadin, junctin, and the ryanodine receptor. Various mutations of the CS2 gene lead to altered Ca^{2+} release and contractile dysfunction contributing to the development of arrhythmias and sudden cardiac death in young individuals affected by catecholaminergic polymorphic ventricular tachycardia (CPVT). Rizzi et al. (2008) have recently developed a transgenic mouse carrying one of the identified CS2 point-mutations associated to CPVT, R33Q, in which a drastic reduction of the mutated protein was observed. In this context, we wanted to: (1) identify when the reduction of CS2 begins; (2) unveil the mechanism involved in the reduction of CS2; (3) verify if other proteins are affected by the presence of the mutated protein; (4) study any modifications in morphology of SR by electron microscopy. We found that (a) mutated CS2 begins to decrease soon after birth in conjuction with the decrease of other proteins of the junctional SR, such as triadin; (b) there is up-regulation of proteins associated to the unfolded protein response (UPR); (c) the pathway activated by UPR is the ATF6-dependent one; d) SR is swollen. In conclusion, the mutation R33Q induces the decrease of CS2 by activation of the UPR and following degradation through proteasome.

P.38

HIF-1α regulates the oxidative capacity of adult rat skeletal muscle

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The aim of this study was to investigate the effects of Hypoxia Inducible Factor-1α (HIF-1α) overexpression in the typical fast myosin heavy chain (MyHC)-expressing/glycolytic muscle, the extensor digitorum longus (EDL), and the slow MyHC-expressing/oxidative soleus of normally active adult rats. Overexpression after somatic gene transfer lead to a decrease in oxidative capacity in both EDL and soleus muscle. Oxidative capacity was measured by histochemical staining for the mitochondrial enzyme succinate dehydrogenase (SDH) on muscle sections. In soleus, a significant SDH reduction of 16% was found in experimental type I/IIa hybrid fibres, with no significant difference found in type I and IIa fibres. This is in conjunction with our previous finding of an increase in hybrid I/IIa fibres in soleus in the same data set. In fast EDL muscle, total SDH was reduced by 25%. This was partly due to a shift in fibertype that we describe elsewhere, but notably, a 17% significant reduction of SDH staining of glycolytic IIb fibres was observed. No significant change was noted in type IIa or IIx fibres. Measurement of stain intensity of a glycolytic enzyme, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is currently being analysed. In conclusion, these results indicate that HIF-1α plays a role in the regulation of metabolic properties in adult muscle.

P.39

3D estimation of the capillary pattern in human masseter muscle

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Skeletal muscles require a well developed capillary network, which supplies fibres with oxygen and removes waste metabolic products. In human masseter muscle capillarization has been evaluated from 2D sections counting capillary profiles per area unit, fibre number, or around the fibre or methods that implied also fibre diameter were applied. Quantitative analysis from 2D sections is problematic and biased, as the total length of capillaries could be essentially underestimated when capillaries are tortous and branched. The aim of this study was to develop immunofluorescent staining of capillaries and muscle fibres in thick sections of human autopsy samples from the anterior superficial part of masseter muscle. The images, captured with confocal microscope served for 3D visualisation of capillaries and muscle fibres. Capillaries were automatically segmented and the result was manually corrected. Quantitative evaluation of capillary network implied the length of capillaries within a unit volume of muscle tissue (L_{cap}/V_{muscle}), length of capillaries supplying individual muscle fibres (L_{cap}/L_{fib}) as well as their course in the muscle estimated by the tortuosity and orientation were performed. Orientation of capillaries in the 3D space was estimated by the degree of anisotropy. The results show differences in capillary pattern of young and elderly persons. To our knowledge this is the first research on human masseter muscles which applies quantification in the 3D space.

P.40

Localization and function of SMPX (Chisel) in rodent skeletal muscle

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Mechanical factors such as stretch are thought to be important in the regulation of muscle phenotype. Small muscle protein X-chromosome (SMPX) is up regulated by stretch in skeletal muscle. Based on cell culture over-expression experiments and staining of isolated murine skeletal and heart muscles, Palmer et al. (2001) hypothesized that SMPX may influence intracellular signalling events, promote a slow fibre type and lead to structural changes in skeletal muscle in vivo. We have used in vivo confocal imaging to study the sub cellular localization of SMPX in skeletal muscle fibres of adult rats and mice, using a SMPX::EGFP fusion protein. It was localized predominantly in repetitive double stripes flanking the Z-disc, and was excluded from all nuclei. Surprisingly, control fibres transfected with EGFP also had a striated pattern, but in contrast to SMPX::EGFP fibres it was denser in the A-band, at the level of myosin. In vivo over-expression of native SMPX in skeletal muscle of adult mice gave no significant changes in fibre type distribution or cross sectional area in extensor digitorum longus, whereas in soleus a shift towards a faster fibre type (23% decrease in type 1) and an increased cross sectional area (11%) was observed. Over-expression results from rats are currently being compiled. Our results indicate that SMPX may not have any major phenotypic effect on fibre type and size in skeletal muscle, and its role may be strictly structural or mechanical.

P.41

The role of Sox6 in skeletal muscle fiber type differentiation

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Our laboratory previously reported that Sox6 functions as a suppressor of slow fiber type specific genes in the developing fetal skeletal muscle. In the absence of Sox6, the suppression of slow fiber specific genes fails to occur, causing the Sox6 null muscle to sustain a slow fiber phenotype as late as P10. To further understand how Sox6 regulates muscle fiber type differentiation, we have been conducting the following experiments. (1) Generate skeletal muscle specific Sox6 knockout to determine whether loss of Sox6 has a lasting effect on muscle fiber phenotype in adult, (2) Identify Sox6 target sequences using ChIP-seq, and (3) Identify Sox6 cofactors in skeletal muscle using yeast two hybrid screening. Here, we will report our recent results of these experiments: (1) Using Myf5-Cre, adult muscle-specific Sox6 null mice were successfully obtained. MyHC isoform mRNA expression in hindlimb muscle was analyzed by qRT-PCR. A significant increase in myh7 (e.g. $\sim$ 3000–4000-fold in EDL), with a concurrent decrease in myh4, was detected. (2) As for Sox6 binding sequences, we have identified 5′ upstream and intron regions of muscle specific genes (e.g. myh7, myh14, Tnnc1, Tnnt1), transcription factors, and histone modification enzymes. (3) An E3 ubiquitin ligase Trip12/ULF has been identified as an interacting protein of Sox6. We have confirmed that Sox6 is poly-ubiquitinated by Trip12 in vitro.

P.42

Propagation of intercellular Ca^{2+} waves in arterial smooth muscle cells

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Smooth muscle cells (SMCs) present in the arterial wall are able to relax and contract to regulate the vascular tone. The contraction is caused by an increase in the SMCs cytosolic calcium concentration ($[Ca^{2+}]i$) in response to the vasoconstrictors present in the vascular system. This increase in the $[Ca^{2+}]i$, and the contraction of SMCs, can propagate as a wave along the arterial axis. To investigate the mechanisms underlying Ca^{2+} wave propagation, we stimulated primary cultured rat mesenteric SMCs (pSMCs), loaded with the fluorescent dye Fluo-4, by local mechanical stimulation. Stimulation evoked two distinct intercellular calcium waves: a fast Ca^{2+} wave with a velocity of several mm/s and a much slower Ca^{2+} wave with a velocity of few tens of μm/s. Using inhibitors of gap junctions and of voltage-operated calcium channels (VOCCs), we show that the fast Ca^{2+} wave was caused by Ca^{2+} influx through VOCCs and depends on the propagation of membrane depolarization. The slow Ca^{2+} wave however, was caused by Ca^{2+} release from the sarcoplasmic reticulum. These results suggest a possible mechanism underlying the propagation of intercellular Ca^{2+} waves in vascular SMCs.

P.43

The effects of VEGF on skeletal muscle phenotype in adult rats

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Vascular endothelial growth factor (VEGF) has been showed to be an important mitogen in both vasculogenesis and angiogenesis. Up regulation of VEGF is seen in both tumor growth and hypertrophy in heart muscle. In the heart it has been suggested that VEGF could contribute to hypertrophy (Tirzui et al. 2007; Shimojo et al. 2007). We have over-expressed the isoform VEGF-a in the fast skeletal muscle extensor digtorum longus (EDL) in adult male Wistar rats, after somatic gene transfer by electroporation. The effects on fibertype, capillarisation, fibre cross sectional area and oxidative capacity were studied. No change in fibre type distribution was observed as judged by immunohistochemistry. Histochemical analyses showed a significant increase in the number of capillaries around 2× and 2b by 9.5 and 30.7%, no change was observed in 2a fibres, but these already have a high number of capillaries. The cross sectional area of 2a and 2b fibers was significantly increased by 12.4 and 9.4% respectively. Measurements of oxidative capacity are under way. Our data suggest that VEGF might not only influence capillarization, but also act back on the muscle fibres themselves as to induce hypertrophy.

P.44

Comparison of electroporation and lipofection for transfer of plasmid DNA into human myoblasts

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One of main goals in vitro research is to develop efficient methods for drug delivery. Effective delivery of genetic material into cells is crucial for application in clinical environment for gene therapy or genetic vaccination. The tissue of choice for gene therapy or DNA vaccination are skeletal muscles, representing 40% of the body mass and which are known to actively participate in the immune response. Genetic material and foreign molecules can be introduced into eukaryotic cells by various ways. Here we compared the efficiency of two different methods for transfer of plasmid DNA: electroporation, where electric pulses are used to permeabilize cell membrane; and lipofection which uses chemical materials (liposomes) as carriers. Electroporation is promising for clinical application while lipofection is auspicious for developing strategies for gene therapy. Plasmid pEGFP coding for green fluorescent protein was transfected into primary human myoblasts either with lipofection (Lipofectamine 2000) or applying high-voltage pulses. Preliminary results show a substantial lower rate (37%) of plasmid DNA lipofection (viability 80%) in comparison to 80% efficiency with siRNA lipofection, this might be probably due to the high size of the plasmid (4.7 kbp). The highest rate of transfection (24%) with electroporation was achieved with 8 × 1 ms electric pulses (viability 37%). Since each of these methods has its own advantages it is important to know which parameters determine efficiency of both methods in vitro as well to understand the mechanism involved in gene transfer in order to optimize in vivo applications of gene electrotransfer or lipofection.

P.45

Muscle fiber diameters of normal human diaphragm

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Diaphragm, the main inspiratory muscle, is highly specialised skeletal muscle. It is rarely selectively affected at neuromuscular diseases. The coastal part of the diaphragm could be examined by EMG at human, but morphological studies of human diaphragm are sparse. Data for diaphragmatic innervation ratio, fiber density and motor unit territories are lacking and a single study about diaphragmatic muscle fiber diameters could be found in Medline (Sauleda J et al. 1998). It was recently demonstrated by quantitative EMG (Podnar S and Resman-Gaspersic A 2008) that diaphragmatic motor unit potentials (MUPs) have lower values of parameters which describe motor unit size (smaller amplitudes, duration, area and smaller value for number of phases and number of turns). Autopsy samples of coastal part of diaphragm bilaterally and of vastus lateralis muscle on the left side were collected from healthy men aged from 30 to 60 years, who died of sudden death. Muscle fiber types were determined by the demonstration of myosin heavy chains by indirect immunoperoxidase method. Slow twitch fibers, fast twitch fibers and intermediate fiber types were determined by commercially available antibodies. Computer aided method for the quantification of muscle fiber parameters was used. Mean diaphragmatic muscle fiber diameters of all types of fibers were significantly smaller compared to vastus lateralis muscle ($P < 0.0001$). No difference between right and left side of the diaphragm was detected. Smaller diaphragmatic muscle fiber diameters could contribute to special characteristics of diaphragmatic MUPs among possible other parameters (diaphragmatic thickness, recording characteristics).

P.46

Different expression of sphingosine 1-phosphate receptor 1 in skeletal muscle in relation to gender, age and use

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Sphingosine 1-phosphate (S1P) is a bioactive signaling lipid that regulates important cellular processes, including proliferation, survival, growth, differentiation and migration. The extracellular action of S1P is mediated by the binding to five high-affinity G protein-coupled receptors ($S1P_{1\text{-}5}$) that generate multiple downstream signals. In skeletal muscle, we demonstrated that $S1P_1$ and $S1P_3$ receptors are localized in the plasma and T-tubule membranes, consistently with a trophic action exerted by S1P in denervated muscle (Zanin et al. Am J Physiol 294:C36–46, 2008). These receptors are also evident at the neuromuscular junction level and in the nuclear membrane. $S1P_1$ and $S1P_3$ are also demonstrated in quiescent satellite cells, whereas $S1P_2$ receptor is only transitorily expressed in activated satellite cells, validating the role of S1P in muscle regeneration (Danieli-Betto et al. Am J Physiol 298:C550–8, 2010). In the present work we analyzed the expression of $S1P_1$ receptor in different muscles and conditions. Western blot analysis shows that rat and mouse muscles express two different $S1P_1$ protein bands, whose relative proportion depends on the muscle type (slow and fast), gender and age of animals. Moreover, denervation, regeneration and electrical stimulation differently affect both the relative expression level and membrane-to nucleus localization of the two $S1P_1$ forms. Funded by PRIN 2008.

P.47

Superoxide flashes in skeletal muscle are produced by discrete arrays of active mitochondria operating coherently

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Reactive Oxygen Species (ROS) constitute most important intracellular signaling molecules. Mitochondria are classically admitted sources of ROS, especially of superoxide anions through the electron transport chain. In the present work, the mitochondria-targeted ratiometric-pericam-mt (RPmt) was used as a specific superoxide biosensor, by appropriate choice of the excitation wavelength. RPmt was transfected in vivo into mouse muscles. Confocal imaging of the isolated muscle fibers reveals spontaneous burst (flashes) of RPmt fluorescence. Flashes correspond to increases in superoxide production, as shown by simultaneous recordings of the fluorescence from MitoSox, a mitochondrial superoxide probe. On average, superoxide flashes are $\sim$20 s-long and yield an amplitude ($\Delta F/F$) of 0.95 ± 0.03. They occur in all subcellular populations of muscle mitochondria.

Strikingly, spatial analysis of the flash pattern over time revealed that arrays of mitochondria work as well-defined superoxide-production-units, active units being adjacent to quiescent ones. Increase of superoxide production at the muscle fiber level appears to involve recruitment of supplemental units with no increase in per-unit production. Altogether, these results demonstrate that superoxide flashes in muscle fibers correspond to physiological signals intimately linked to mitochondrial metabolism. They also suggest that superoxide may self-enhance its own production by activating quiescent mitochondria.

P.48

Software for muscle fibre type classification and analysis

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Fibre type determination requires a long series of differently stained muscle sections. The manual identification of individual fibres through the series is tedious and time consuming. We present the software that enables: (i) an image registration which includes the adjusting of the individual fibre position and its identification through a series of differently stained muscle sections, (ii) muscle fibre classification and finally (iii) quantitative analysis. The data output of the system includes numerical and areal proportions of fibre types, their average diameter and optical density (grey level) of the final product in each registered fibre. The muscle fibre type can be determined stepwise analysing one set of stained sections. However, the software enables the analysis of any number of subsequently added staining reactions to already defined muscle fibre profile. The system is semiquantitative, flexible, and user friendly. It can be applied in skeletal muscle research whenever the information about the muscle fibre type pattern is needed. The study was financially supported by the Slovenian Research Agency and the Ministry of Education, Youth and Sports of the Czech Republic (KONTAKT grant No. MEB090910 and grant No. LC06063).

P.49

PGC-1β's role in adult muscle plasticity in mice

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Plastic changes in muscle fibers include the activation of genes involved in mitochondrial biogenesis, oxidative phosphorylation, and fast and slow isoforms of contractile proteins. Recently, the PPAR gamma co-activator (PGC)-1β has been proposed to take part in regulating such processes in transgenic animals by altering oxygen capacity and myosin heavy chain expression in individual muscle fibers. However, it is difficult to know if the observed effects reflect a true adult plasticity, or is an effect of PGC-1β over-expression during myognesis. We compared the endogenous expression patterns of PGC-1β in both fast and slow adult muscles and investigated the effect of forced PGC-1β expression on fiber phenotype after somatic gene transfer in the adult. PGC-1β expression was measured by subcellular protein fractionation and Western blotting, and we found that PGC-1β was expressed 36-fold higher in nuclei from extensor digitorum longus (EDL) than nuclei from soleus. PGC-1β was not detected in the cytosolic fraction of either muscle. In vivo over-expression studies using the fusion protein Flag-PGC-1β resulted in a shift towards a slower fiber phenotype in the fast EDL, by increasing the amount of 2× fibers at the expense of 2b fibers. No significant alterations were seen in the slow soleus. This supports the theory that PGC-1β takes part in muscle plasticity and induces 2× fibers, in adult mice.

P.50

Mice deficient in the serum- and glucocorticoid-inducible kinase SGK1 show reduced muscle force and an increased fraction of type IIB fibres

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The serum- and glucocorticoid-inducible kinase SGK1 participates in many physiological functions, such as gene expression, metabolism and cellular transport. Since the enzyme is considerably expressed in skeletal muscle, we investigated a possible role of SGK1 for muscle structure and function. Histological analyses and force measurements were performed with hindlimb muscles and diaphragms from 100 days old wildtype and SGK1 deficient mice ($sgk1^{-/-}$). Muscle mass, kinetics of twitches and tetanic force as well as fatigue development were not altered in soleus muscles and diaphragms of $sgk1^{-/-}$ mice. However, specific muscle force was significantly reduced in $sgk1^{-/-}$ muscles (force reduction: 20% for M. soleus and 25% for diaphragm, $n = 7$ for each muscle). The histological appearance of muscles from $sgk1^{-/-}$ mice was overall intact. The area filled with connective tissue was in soleus muscles on average 3.5% for $sgk1^{-/-}$ animals (wildtype 4%). Myosin ATPase staining revealed a change in fibre type composition in $sgk1^{-/-}$ mice. In soleus muscles the area covered by type I fibres was $43 \pm 4\%$ for the $sgk1^{-/-}$ animals (wildtype: $48 \pm 2\%$). In contrast, the area covered by type IIB fibres was significantly increased. We conclude that the lack of SGK1 does not lead to pronounced changes in muscle structure and function. However, the enzyme seems to be involved in muscular gene expression and thereby affects maximal muscle force and fiber type composition of skeletal muscles.

P.51

Akt-mTOR signaling is preferentially activated by resistance exercise, while AMPK signaling is activated by both endurance exercise and resistance exercise in training-accustomed individuals

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An AMPK-Akt "switch" signaling mechanism has previously been suggested to explain adaptations to low-frequency versus high-frequency electrical stimulation in rat muscle. Similar signaling selectivity to explain adaptations to endurance versus resistance exercise in humans seem equivocal, as a larger degree of signaling overlap has been observed between exercise modalities, but which may partly rely on inherent limitations of such human studies. Here, we add to the hypothesis in a study on training-accustomed individuals. 20 untrained male individuals were randomly divided in endurance, resistance or non-exercise control groups. The exercise groups completed 10 weeks of progressive training followed by a single-bout exercise trial. Muscle biopsies were harvested prior to and at 0, 2½, 5 and 22 h after single-bout exercise and analysed for markers of AMPK and Akt signalling. Overall upregulation was observed for pAkt, pPAS AS160, pmTOR, pGSK3 and peIF4E after resistance exercise, but not endurance exercise. Overall upregulation of pAMPK, pHDAC5, pCREB and pACC was observed after endurance exercise, with a similar overall tendency after resistance exercise and with no differences between groups. The current results suggest that Akt-mTOR signaling respond preferentially to a hypertrophy-inducing exercise stimulus, whereas AMPK-signaling is more equally induced by contractility or the metabolic costs of differentiated exercise.

P.52

Sarcomeric M-band alterations characterize heart pathology

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The M-band is believed to cross-link the thick filaments in the sarcomere. Its main components are three related proteins from the myomesin family. Their expression was shown to correlate with the contractile parameters of different muscles. To investigate the M-band alterations in heart disease, we analyze the expression of myomesins in mouse and human cardiomyopathy. Cardiac function is assessed by echocardiography and compared to the myomesins expression evaluated with RT-PCR, Western blot, and immunofluorescence. We found that disease progression in two mouse models for DCM is accompanied by specific M-band alterations. The EH-myomesin isoform is strongly up-regulated, correlating to cardiac function ($R = -0.86$). In following, we have analyzed the myomesins expression in heart biopsies obtained from DCM patients, DCM patients supported by a left ventricular assist device (LVAD), HCM patients and controls. qRT-PCR reveals that the EH-myomesin isoform is up-regulated 41-fold ($P < 0.001$) in the heart of DCM patients compared to control patients. The EH-myomesin expression in LVAD-supported and HCM hearts is comparable to control. The EH-myomesin is upregulated in a cell-specific manner, leading to a higher heterogeneity of the myocytes cytoskeleton. We suggest that EH-myomesin upregulation denotes an adaptive remodelling of the sarcomere cytoskeleton in the dilated heart and might serve as a universal marker for DCM progression in mouse e and human myocardium.

P.53

Connecting cardiomyocytes: cytoskeletal organisation at the intercalated disc

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Cardiomyocytes connect at their ends through the intercalated disc (ID), a complex region of membrane folded around thin and intermediate filaments which incorporates mechanical, electrical and signalling domains. We have previously shown that both spectrin and Protein 4.1R, which links the sub-membrane spectrin/actin net to membrane proteins, are present at the intercalated disc. In order to understand the role of the spectrin-associated cytoskeleton at the intercalated disc the organisation of these proteins has been investigated in more detail. Using immunofluorescence the location of αII spectrin and Protein 4.1R in relation to the structural domain proteins, β-catenin (adherens junction) and connexin43 (gap junction), and the signalling proteins, sodium calcium exchanger and sodium potassium ATPase, have been studied. We find that their position does not coincide with the structural domains. Rather they are overlap more with the signalling proteins. These results are consistent with the idea that 4.1 proteins can bind to membrane proteins and thus influence their function, and the observation that in a 4.1R ko mouse the calcium signalling process in the heart is altered. The spectrin rich regions of the ID membrane are 'unstructured' compared to the cell–cell junctions such as the gap junction. To understand the relationship between the structural domains and the unstructured membrane regions in the ID, electron tomographic reconstructions of IDs have been carried out.

P.55

Regionalized ROS production depresses contractile function in ischemic heart failure: preventive effect of a chronic antioxidant therapy

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Ischemic heart failure (HF) is characterized by regionalized contractile alterations resulting in loss of transmural contractile gradient across left ventricular free wall. We aimed to determine whether mitochondrial dysfunction could be involved in the HF contractile defects. Twelve weeks after left coronary artery ligation (MI), NADH homeostasis measured in cardiomyocytes isolated from subendocardial (ENDO) was altered compared with sham rats, and correlated with decreased activity of both complex I and IV of the electron transport chain. Generation of superoxide anion using MitosoxRed dye was increased twofold in MI rats, associated with decreased antioxidant defenses (superoxide dismutase and catalase). Incubation with the antioxidant *N*-acetylcystein (NAC) for 1 h restored most of cell shortening in MI myocytes by restoring the Ca^{2+} homeostasis and the properties of the contractile machinery properties. We showed that the beneficial effect of NAC was mediated by reducing PKA activity that increased during HF, and thus preventing the hyperphosphorylation of proteins such as troponin I and the

ryanodine receptors. Chronic treatment with NAC (4 weeks) improved in vivo contractility by improving excitation–contraction coupling in myocytes. In conclusion regionalized HF-associated mitochondrial dysfunction is responsible of ROS production in ENDO layer that contribute to the loss of transmural contractile gradient and global contractility mediated by PKA activation.

P.56

Role of Rho-associated kinase in myofibrillogenesis and contractility of cardiomyocytes

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Rho-associated kinase (ROCK) is a serine/threonine protein kinase. More than 20 targets of ROCK are identified including cytoskeleton proteins, myosin light chains, myosin phosphatase and LIM kinase that plays important role in actomyosin structure and function in cells. Two members of the ROCK family, ROCK1 and ROCK2, have been identified thus far. Recent evidence suggests that these protein kinases are involved in cardiac development. However, the mechanisms of ROCK participation in heart development remain largely unclear. In order to test a hypothesis that ROCK is involved in myofibrillogenesis in developing heart we have shown that Y-27632, an inhibitor of ROCK, delays sarcomere assembly in cultured rat neonatal cardiomyocytes pretreated with angiotensin II. Y-27632 affects the beat rate of cardiomyocytes but this effect is only observed at high cell density and, therefore, seems to be related to the formation of gap junctions between adjacent cardiomyocytes. Consistent with this suggestion we established that ROCK2 is localized in myofibrillar Z-discs of human myocardium as well as in intercalated discs enriched in gap junctions. We propose that ROCK participates in maturation of myocardial contractile system through phosphorylation of its molecular targets in Z-discs and in intercalated discs.

P.57

Presence, sub-cellular localization and function of Homer1a in cardiac myocytes

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The scaffolding proteins Homers modulate intracellular Ca^{2+} signalling. Both in neuronal and smooth muscle cells the splice variant Homer1a disassembles signalling complexes mediated by other Homer proteins. We investigated the presence, sub-cellular distribution and function of Homer1a in cardiac muscle. Homer1a is constitutively expressed in cardiac muscle of both mouse and rat. The content of Homer1a is considerably larger in heart as compared to that of skeletal muscle. As judged by confocal immunofluorescence microscopy, Homer1a co-localizes with RyR2, involved in excitation–contraction (E-C) coupling, and is closely juxtaposed to the perinuclear IP3RII, putatively involved in Ca^{2+}-dependent gene activation [excitation-transcription (E-T) coupling]. In mouse neonatal myocytes, over-expression of Homer1a increases amplitude and duration of perinuclear, IP3R-dependent Ca^{2+} puffs. In HL-1 cells, the hypertrophic agonist norepinephrine induces rapid Homer1a over-expression and its concentration in perinuclear domains. Thus, in heart, Homer1a shows a peculiar sub-cellular localization compatible with its regulatory role on both IP3RII and RyR2 and its potential role in both E-C and E-T coupling. Homer1a may be one of the molecular modulators of hypertrophy since (i) its content increases during the early stages of cardiac hypertrophy, and (ii) it affects perinuclear IP3-dependent Ca^{2+} release, which in turn regulates gene activation.

P.58

Hypertrophic cardiomyopathy (HCM)-related action potential remodeling: impact on contractile function of human septal samples from diseased patients

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We had previously found that prolongation of action potential duration (APD) of HCM human myocytes is accompanied by significant reduction of repolarizing currents. Here we investigate whether changes of sarcolemmal Ca^{2+} fluxes also contribute to prolong APD and affect contractile performance of HCM tissue. Cardiomyocytes were enzymatically dissociated and intact trabeculae dissected from samples of tissue harvested from HCM patients undergoing septal myectomy. Septal tissue of patients undergoing aortic valve surgery was used as control. $I_{Ca\text{-}L}$ amplitude and kinetics were unchanged but NCX expression was increased five-fold in HCM compared to control cells. Ranolazine (10 μM), a specific blocker of $I_{Na\text{-}late}$, reduced APD by more than 30% in HCM cells while it had no effect on control myocytes, suggesting an up-regulation of $I_{Na\text{-}late}$ in HCM tissue. Ranolazine also reduced Ca^{2+} transient amplitude and duration; in addition, it accelerated Caffeine-induced Calcium transients decay of HCM cells suggesting increased Ca^{2+} extrusion through NCX. When applied to isometrically contracting HCM trabeculae ($n = 5$), ranolazine (i) significantly reduced peak twitch tension, (ii) reduced diastolic tension at high stimulation frequency and (iii) tended to speed up relaxation. Combination of NCX over-expression and intracellular Na^+ accumulation due to increased $I_{Na\text{-}late}$ may promote a large increase of Ca^{2+} entry through NCX reverse mode in HCM myocardium. This may contribute to prolong APD and increase Ca^{2+} transient amplitude and duration. By reducing intracellular Na^+ overload, Ranolazine potentiates Ca^{2+} extrusion trough NCX direct mode promoting negative inotropic effects but significantly ameliorating diastolic function. Telethon grant GGP07133 & EU STREP-Project BIG-HEART 241577.

P.59

Altered relaxation kinetics and contractile phosho-proteome in a β-MHC model of congestive heart failure

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Background: Congestive heart failure (CHF) is a syndrome characterized by the depression of cardiac pump function such that the output of the heart is unable to provide the body with sufficient blood supply. The molecular mechanisms underlying this syndrome are incompletely understood.
Hypothesis: Rate of cross-bridge cycling is depressed in CHF and transient post-translational modifications of the contractile proteins regulate the mechanics.
Methods: We established a 3 stage chronic pressure overload β-MHC model of CHF by surgically implanting AngII pumps (400 ng/kg/min)/saline pumps (0.9%) in female Dunkin Hartley Guinea pigs weighing 400 g, for 12 weeks. Following 12 weeks of treatment, LV samples were frozen in liq. N2. Single myofibrils were prepared by mechanical dissociation and subsequently attached between two glass microneedles that were positioned on the stage of an inverted phase-contrast microscope. 2-D DIGE gels using Cy3 and Cy5 dyes were run to study the post-translational modifications within the groups.
Results: Single myofibril studies indicate slow relaxation kinetics in the failure group. In addition, 2-D DIGE gel analysis revealed shifts in the phosphorylation profiles of the contractile proteins MyBP-C, MLC-2, TnT and TnI.
Conclusions: We conclude that altered relaxation kinetics underlie, in part, the decreased pump function that is seen in this experimental model of CHF and that this phenomenon may be caused by maladaptive contractile protein phosphorylation.

P.60

Age-related decline in contractile function of ventricular myofibrils from HCM patients carrying MYBPC3 mutations

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Cardiac Myosin Binding Protein C gene (MYBPC3) is the most frequent target of sarcomeric protein mutations associated to Hypertrophic Cardiomyopathy (HCM). Since a few years we have been examining the functional impact of these mutations directly in the cardiac sarcomeres of HCM patients undergoing septal myectomy. We use the single myofibril technique (Belus et al. 2008) to investigate Ca^{2+}-sensitivity, mechanics and kinetics of contraction and relaxation of myofibrils isolated from myectomy samples of patients of different ages (range 18–68 year) carrying different MYBPC3 mutations. Preparations, mounted in a force recording apparatus (15°C), are Ca^{2+}-activated (pCa 6.50–4.50) and fully relaxed (pCa 8) by rapid (<10 ms) solution switching. Ca^{2+}-sensitivity of tension is always increased in MYBPC3 mutant myofibrils compared to controls. Maximal isometric tension is always reduced and full tension relaxation upon Ca^{2+} removal is often impaired in the myofibrils from MYBPC3 mutant patients of any age. The rate constant of active tension generation following maximal Ca^{2+} activation (k_{ACT})—as well as the rate of tension redevelopment following mechanical perturbations (k_{TR})—are significantly faster in the myofibrils from young MYBPC3 mutant patients (<40 year) as compared to controls. At older ages (>40 year) k_{ACT} and k_{TR} of the mutant myofibrils progressively decline to levels that are the same as the controls or below the controls. Consistently, on a large HCM cohort we found that patients harbouring MYBPC3 mutations show an age-related decline in left ventricular function and are more likely to develop heart failure compared to other HCM patient groups. Acknowledgement: EU STREP-Project BIG-HEART 241577; Telethon GGP07133.

P.61

Single amino acid substitution at conserved residue confers unique kinetic properties to mouse alpha-cardiac myosin

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Loop 1 and residues around the nucleotide binding pocket are likely regulators of the turnover kinetics of myosin heavy chain (MYHC). Species-dependent sequence variations in regions near the nucleotide binding pocket may confer different turnover kinetics via interactions with loop 1 to modulate the coordination of MgADP in the nucleotide binding pocket. We screened various MYHC genes to identify regions that may account for functional differences between MYHC isoforms with sufficient charge density to provide complementary binding for loop 1. One region was indentified (residues 323–351) and revealed that mouse alpha-MYHC expressed Ser rather than the Gly residue at position 341 commonly observed in other mammalian cardiac myosins. We generated mice expressing homozygous S341G alleles for a cardiac alpha-MYHC to test the hypothesis that the S341G mutation would reduce ATPase activity by slowing the rate of MgADP release in murine alpha-MYHC, thereby slowing myocardial contraction kinetics. Po and pCa50 did not differ significantly between the WT and S341G skinned myocardium. However, ktr, kdf, and Vo were significantly slower in maximally activated preparations isolated from S341G myocardium. Furthermore, S341G mutant myosin demonstrated a significant reduction in the rate of ADP dissociation. These results indicate that the unique Ser substitution at residue 341 in alpha-MYHC plays a critical role in conferring very fast turnover kinetics in murine myosin.

P.62

Platform CARDIEX : a tool for functional exploration in small animals

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CARDIEX platform offers a unique service, ranging form the generation of animal models to highly specialized functional analyses in the field of cardiovascular, metabolic, muscular, digestive, respiratory and cancerous pathologies. CARDIEX belongs to the national platform network IBISA, is connected with IFR26 and involves multi-disciplinary teams from Inserm UMR915, UMR913, UMR892 and Cancéropôle Grand Ouest. CARDIEX provides academic research group and biopharmaceutical companies with a large panel of technologic tools and scientific expertise to analyze new therapeutic pathways from validation of interest targets to evaluation of efficacy and the side-effects of drugs. It offers small animal models which mime human pathologies as dyslipidemia, diabetes, arterial hypertension, restenose or cardiac, neuro-digestive or muscular pathologies. These models involve transgenic or no-transgenic animals, on which we can test therapeutic interest molecules. In collaboration with In-Cell-Art company, CARDIEX also offer R&D project of animal model generation using somatic transgenesis or genic immunization. Its potential, when coupled to appropriate phenotyping activity, to dissect the role of each protein in (patho)physiological and biochemical pathways, making it an unparalleled vehicle for functional genomics

studies. CARDIEX therefore offers access to equipments, functional exploration services for small animals and R&D projects.

P.63

A study of hypertrophic cardiomyopathy (HCM) mutations of tropomyosin (Tm) in thin-filament reconstituted bovine myocardium (cardiac muscle fibers)

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HCM mutants (V95A, D175N, E180G) of Tm were examined by thin-filament extraction/reconstitution technique using bovine myocardium. Effects of Ca^{2+}, ATP, Pi and ADP on force and its transients were studied. All mutants showed significantly larger relaxed tension (0.29–0.42) than WT (0.12 ± 0.02). At pCa 4.66, active tension was decreased in V95A (1.10 ± 0.07) and D175N (1.12 ± 0.08) compared to WT (1.52 ± 0.09) or E180G (1.48 ± 0.11). pCa50 was larger in V95A (6.20 ± 0.06) and E180G (6.49 ± 0.02) than WT (5.85 ± 0.03) or D175N (5.88 ± 0.05). The cooperativity was decreased in mutants (1.70–1.87) compared to WT (2.79 ± 0.26). 5 equilibrium and 4 rate constants were obtained from sinusoidal analysis. V95A showed reduced $K0$ (ADP association, 14.8 ± 0.9 mM^{-1}), $K1$ (ATP association, 0.86 ± 0.16 mM^{-1}), $K2$ (cross-bridge detachment, 0.93 ± 0.06), $K5$ (Pi association 0.22 ± 0.04 mM^{-1}), k-2 (30.3 ± 1.2 s^{-1}), and $k4$ (force generation, 8.32 ± 0.62 s^{-1}) compared to WT ($K0 = 24.5 \pm 2.7$ mM^{-1}; $K1 = 1.76 \pm 0.35$ mM^{-1}; $K2 = 1.37 \pm 0.13$; $K5 = 0.58 \pm 0.13$ mM^{-1}; k-2 = 19.4 ± 1.2 s^{-1}; $k4 = 5.93 \pm 0.65$ s^{-1}). D175N showed decreased $K5$ (0.26 ± 0.04 mM^{-1}) and $k2$ (33.0 ± 2.3 s^{-1}) compared to WT (25.6 ± 1.9 s^{-1}); E180G showed increased $K0$ (33.2 ± 1.3 mM^{-1}). None of the mutants showed any significant change in $K4$. The number of strongly attached cross-bridges was not different among 4 Tms. We propose that impaired relaxation, decreased active tension, and increased pCa50 increase the myocyte stress resulting in hypertrophy, and contribute to the severity of the symptom.

P.64

Protein kinase C α phosphorylation of human cardiac troponin exerts a negative lusitropic and inotropic effect in human cardiomyocytes

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Alpha-adrenergic receptor stimulated protein kinase C (PKC) is able to modify cardiac contractile function via phosphorylation of several myofilament proteins. Previously, we reported a decrease in Ca^{2+}-sensitivity of force (pCa_{50}) of failing cardiomyocytes incubated with PKCα. This study aimed to determine the specific effects of PKCα-mediated phosphorylation of cTn on myofilament function in human failing cardiomyocytes. Endogenous cTn in failing cardiomyocytes was partially exchanged (up to 70%) with recombinant human cTn(DD) in which the PKA sites on cTnI Ser23/24 are mutated into aspartic acids to rule out cross-phosphorylation of the PKA sites. Moreover cTn(DD) complex was used pre-treated with PKCα (cTn(DD + PKCα)). Isometric force was measured at different [Ca^{2+}] in single permeabilized cardiomyocytes from failing left ventricular tissue. Failing cardiomyocytes exchanged with cTn(DD) complex showed a pCa50 of 5.50 ± 0.03. Exchange with the cTn(DD) complex incubated with PKCα showed an increased Ca^{2+}-sensitivity ($pCa50 = 5.59 \pm 0.02$). Surprisingly, subsequent incubation with PKCα reduced pCa_{50} to 5.45 ± 0.02. The maximal force generating capacity (F_{max}) was significantly reduced by exchange of cTn(DD + PKCα)(17.1 ± 1.9 kN/m^2) when compared with the cTn(DD) group (25.7 ± 2.7 kN/m^2). Subsequent incubation with PKCα did not restore Fmax. In conclusion, PKCα-mediated phosphorylation of cTn increases the Ca^{2+}-sensitivity but decreases the maximal force resulting in complex alterations in myofilament function in human cardiomyocytes.

P.65

PKG-mediated phosphorylation of titin N2-B unique sequence is a conserved mechanism in mammalian heart

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Titin is a major determinant of myocardial elasticity and passive stiffness. Titin stiffness can be lowered by cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG)-mediated phosphorylation of the cardiac-specific N2-B unique sequence (N2-Bus). A serine residue in the human cardiac N2-Bus was identified as a PKG and PKA phosphorylation site. However, this site is not conserved among species, thus questioning the physiological relevance of titin phosphorylation in commonly used animal models of heart failure. Using online prediction routines we identified putative PKG-targeted phosphorylation sites in dog and mouse/rat N2-Bus. PKG-mediated phosphorylation of the predicted sites was tested by incubation of recombinantly expressed N2-Bus, and NH_2-terminal and COOH-terminal peptides of dog and mouse N2-Bus with PKG. Phosphorylation of titin N2-Bus was shown in peptides from both investigated species using phospho-specific protein stain or autoradiography. We found that PKG phosphorylates an NH2-terminal fragment of the dog N2-Bus, and a COOH-terminal fragment of the mouse/rat sequence. We presume that phosphorylation of a single site in the N2-Bus is likely to cause a similar reduction in titin stiffness as observed for the human titin sequence, regardless to the exact site of phosphorylation. Our results suggest that PKG-mediated phosphorylation of titin N2-Bus is an important regulatory event not only in human but in all mammalian hearts.

P.66

Distinct roles of ADF/cofilin isoforms in sarcomere assembly and maturation

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Actin filaments form various structures that play an essential role in a large number of cell biological processes in all eukaryotes. The actin

cytoskeleton in non-muscle cell is highly dynamic. In contrast, the actin filaments in muscle cells are assembled into sarcomeres and are believed to be significantly less dynamic compared to most actin structures in non-muscle cells. However, many proteins promoting actin dynamics in non-muscle cells are expressed also in muscles. Thus, it is possible that the muscle-specific isoforms of these proteins promote actin dynamics differently from their non-muscle counterparts. ADF/cofilins are central actin-binding proteins that enhance turnover of actin filaments by severing and depolymerizing F-actin. This class of proteins consists of three isoforms in mammals; cofilin-1, cofilin-2 and ADF. Our Western blot analysis revealed that cofilin-1 and cofilin-2 are expressed in cultured rat cardiomyocytes and that the ratio between these proteins changes during the maturation of cardiomyocytes. Studies using isoform specific antibodies against cofilin-1 and cofilin-2 revealed distinct localization patterns of these proteins in cultured cardiomyocytes. Biochemical analyses revealed that cofilin-1 and cofilin-2 display different thermal stability, affinity to actin filaments, and effects to actin dynamics. Together, these data suggest that cofilin-1 and cofilin-2 play distinct roles in regulation of actin dynamics in heart muscle cells.

P.67

Insulin signaling regulates cardiac titin properties in heart development and diabetic cardiomyopathy

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Isoform-switching of titin is a main mechanism for adjusting passive myocardial stiffness. Evidence suggested that thyroid-hormone signaling via the phosphoinositol-3-kinase (PI3K)/AKT pathway regulates titin-isoform composition in cardiomyocytes. Here we hypothesized that insulin, via PI3K/AKT, alters titin-isoform composition and titin-based stiffness. We also checked for insulin-induced changes in titin phosphorylation. In embryonic rat cardiomyocytes cultured with insulin for 7 days, the mean proportion of the stiff N2B-titin isoform significantly increased from 53% in controls to 65% in insulin-treated cells. The insulin-dependent isoform shift was blocked by PI3K-inhibitor, LY294002, suggesting involvement of the PI3K/AKT pathway. Titin phosphorylation was increased by the insulin-treatment. The impact of insulin-deficiency in vivo was studied in streptozotocin-treated (STZ) rats as a model of diabetes mellitus type-1. STZ rats developed cardiac hypertrophy, left ventricular fibrosis, and elevated glucose levels. The mean proportion of N2B-titin was significantly decreased from 86% in controls to 78% in STZ hearts. Titin phosphorylation levels were unchanged. Mechanical measurements on skinned cardiac fibers showed minor passive stiffness modifications in STZ myocardium. We conclude that insulin signaling regulates titin-isoform composition and titin phosphorylation in embryonic cardiomyocytes and could contribute to altered diastolic function in diabetic cardiomyopathy.

P.68

Protein phosphatase-5 is a novel player in the N2B-titin spring-associated signaling complex important for the cardiac hypertrophic response

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Titin's elastic I-band region is a hotspot for protein–protein interactions, next to it being important for myocyte extensibility and passive stiffness. In the heart, titin-based stiffness is lowered by PKA- or PKG-dependent phosphorylation of the cardiac titin N2B-unique sequence (N2Bus). However, no protein phosphatase(s) acting on this titin region had been known. We carried out a yeast-2-hybrid screen using the human N2Bus ("bait") and a human cDNA library ("prey") and detected the catalytic domain of the serine/threonine specific protein phosphatase-5 (PP5c) as a binding partner of the N2Bus. This interaction was verified in GST-pulldown assays and immunofluorescence stainings of neonatal rat cardiomyocytes. Autoradiography experiments showed that recombinant PP5 dephosphorylates recombinant, PKG-phosphorylated, N2Bus. Further, PP5 bound more strongly to the (PKA- or PKG-) phosphorylated N2Bus than to the unphosphorylated N2Bus, suggesting this phosphatase is an antagonist to PKA/PKG at the N2Bus. Phosphorylation of both the N2B and N2BA titin isoforms in human heart tissue was reduced by PP5. Passive force measurements on permeabilized single human cardiomyocytes unexpectedly revealed a drop in passive stiffness owing to PP5. These experiments suggest PP5 is part of a cardiac-specific signaling node at the N2Bus.

P.69

Redox state of cardiac myofibrillar proteins in streptozotocin-induced diabetic rats

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It has been shown that reactive oxygen species production is increased in diabetic heart, and increased oxidative stress may contribute to contractile dysfunction of cardiac muscle. However, the mechanisms underlying the impaired function remain incompletely understood. In the present study, we tested the hypothesis that diabetes-induced alterations in contractile function would stem from oxidative modification of myofibrillar proteins. Diabetes was induced by intraperitoneally single injection of streptozotocin (50 mg/kg body wt) in male Wistar rats (250–300 g). Six weeks post-injection, the protein amounts of myosin heavy chain (MHC) and actin and the content of carbonyl and free sulfhydril (SH) groups were determined in cardiac muscle. Blood glucose levels in diabetic rats were elevated approximately threefold. A trend towards a decrease was seen for the SH group content in cytosolic fraction. The amounts of MHC and actin were unchanged. Immunoblotting with anti-dinitrophenyl antibody did not reveal significant changes in the carbonyl group content contained in MHC, actin or total myofibrillar proteins. These results suggest that oxidative modification of proteins may not associated with disturbances in myofibrillar function from diabetic heart.

P.70

Chronic activation of β^2-adrenoceptor signalling reduces cardiac mitochondrial protein synthesis and activity of oxidative enzymes in mice

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Chronic administration of the β^2-adrenoceptor agonist, formoterol, increases skeletal muscle mass and function but impairs cardiac relaxation via reduced SR Ca^{2+}-ATPase protein and activity, which is likely to cause metabolic and morphologic aberrations in the heart. We hypothesized that chronic formoterol treatment in mice would reduce mitochondrial protein synthesis, reduce activity of oxidative enzymes, and increase apoptosis in cardiomyocytes. The rate of mitochondrial protein synthesis in the heart was significantly lower ($\sim 13\%$, $P < 0.01$) following 28 days of chronic treatment with formoterol. Citrate synthase activity in the heart was 17% lower in formoterol treated mice. Hydroxyacyl-CoA dehydrogenase activity tended to be lower ($\sim 11\%$, $P = 0.10$) in formoterol treated animals. Several markers of mitochondrial biogenesis and function were also significantly reduced while no alteration in the expression of key-regulatory proteins of apoptosis and protein breakdown was observed. These findings highlighting the fact that the therapeutic potential of β-agonists for skeletal muscle wasting conditions will only be realised if these less desirable effects on the heart can be obviated, perhaps through differential signalling mechanisms on skeletal and cardiac muscle. Supported research grants from the Association Française contre les Myopathies and the National Health & Medical Research Council of Australia (Grant: 509313). BL was supported by the Swiss National Science Foundation.

P.71

Adipocyte fatty-acid binding protein contributes to heart failure by suppressing cardiomyocyte contraction

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The increasing prevalence of obesity has become a serious health problem, since obesity has been strongly associated with several harmful cardiovascular disorders such as heart failure. Despite several investigations the pathophysiological mechanisms involved remain unclear. Latest studies have emphasized the importance of adipose tissue as a highly endocrine organ which releases a wide variety of bioactive substances. In this context we have recently showed that adipose tissue exerts highly potent cardiodepressant activity with an acute effect directly on cardiomyocytes contraction.

In the present study, we have identified this cardiodepressant factor as adipocyte fatty-acid binding protein (FABP4). FABP4 is a member of the intracellular lipid-binding protein family and is predominantly expressed in adipose tissue. Although cytoplasmic FABP4 is involved primarily in intracellular lipid trafficking, recent studies have reported a novel role for FABP4 in the pathogenesis of atherosclerosis and diabetes mellitus. In this study we showed that FABP4 represents 1.8–8.1% of total protein secreted by adipocytes in extracellular medium used in our experiments, and directly depresses shortening amplitude as well as intracellular Ca^{2+} peak in a dose dependent manner within a few minutes in isolated rat cardiomyocytes. These data indicate that the release of FABP4 from adipocytes may be directly involved in the pathogenesis of cardiac contractile dysfunction of obese patients.

P.73

Hypertrophic cardiomyopathy caused by mutations in the essential myosin light chain gene: analysis of the pathomechanism

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Hypertrophic cardiomyopathy (HCM) is a disease of the myocardium and associates with mutations in different sacromeric proteins. Cardiac contraction is performed by the interaction of actin with the motor domain of the heavy chains of type II myosin (MHC).

The lever arm domain of myosin contains the IQ motifs which forms the attachment site for the essential light chain (ELC). The ELCs are thought to stabilise the alpha-helix of the hydrophobic myosin neck and to adjust the rigidity of the lever arm.

The human ventricular essential light chain (hVLC1) contains an actin and a myosin binding site. Five mutations in the ELC gene that are linked with HCM are published until today (R154H, M149V, E143K, A57G and E56G). In this study we want to investigate the related molecular pathomechanism. Binding studies were performed between the mutated/wildtype hVLC1 and the ßMHC (aa 664–915) by using surface plasmon resonance technology. We expressed recombinant ßMHC protein which contained an alanin scan to remove the RLC binding domain. Using ultracentrifugation and cd-spectrometry we ensured the correct folding of the recombinant proteins. Our studies revealed a significant decrease in the binding affinity to ßMHC for the hVLC1 mutations compared to the wildtype protein, especially for the E56G mutation. For further studies skinned fiber experiments and transgenic mouse lines are planned. Thereby force generation and changes of the cardiac contractile parameters will be analysed.

P.74

Uncoupling of TnI phosphorylation from decreased thin filament Ca^{2+}-sensitivity can be triggered by mutations and post-translational modifications

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We measured contractility by IVMA and determined the effect of troponin I phosphorylation on Ca^{2+}-sensitivity. With normal heart muscle Ca^{2+} sensitivity is decreased 2.5-fold upon phosphorylation. With mutations in actin and troponin C causing HCM and DCM we found that the Ca^{2+}-sensitivity was independent of the level of phosphorylation. With ACTC E361G DCM mutation EC_{50} TnP/TndP $= 1.0 \pm 0.1$ $n = 4$; with the TNNC1 G159D DCM mutation EC50 TnP/TndP $= 1.2 \pm 0.1$, $n = 5$; with TG mice expressing the ACTC E99K HCM mutation at 50% EC_{50} TnP/TndP $= 1.14 \pm 0.06$ $n = 4$; in human biopsy with the same mutation EC50 TnP/TndP $= 0.92 \pm 2.2$ $n = 3$. This uncoupling must have been caused by the mutation.

In addition we have studied septal myectomy samples from patients with hypertrophic obstructive cardiomyopathy. In every case the Ca^{2+}-sensitivity was uncoupled from troponin phosphorylation (e.g. sample MM, EC_{50} TnP/TndP $= 1.08 \pm 0.24$, $n = 8$). In this case uncoupling is not due to a mutation and is presumed to be due to some post-translational modification of the troponin. We conclude that the allosteric interaction between the N-terminal peptide of

troponin I and the N terminal lobe of TnC that is released by phosphorylation of troponin I ser 22/23 is a highly labile property that can be uncoupled by mutations in any component of the thin filament associated with cardiomyopathy or by post-translational modifications. The possibility that uncoupling occurs in vivo in other pathological conditions should be considered.

P.75

Analysis of human cardiac MyBP-C phosphorylation in situ using phosphate affinity SDS-PAGE

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In vitro studies indicate that cMyBP-C can be phosphorylated at four different sites (serines 273, 282, 302 and 307 from the mouse sequence). Little is known about the level of cMyBP-C phosphorylation or which sites are phosphorylated in normal heart muscle. We have used phosphate-affinity SDS-PAGE together with a non-specific anti-cMyBP-C antibody and a range of phosphorylation site-specific antibodies for the main sites to make a detailed quantitative analysis of cMyBP-C phosphorylation in heart tissue in situ. In human heart myofibrils, five bands were seen corresponding to 0, 1P, 2P, 3P and 4P. We have found that cMyBP-C is highly phosphorylated in normal human heart or mouse heart with tris- and tetra-phosphorylated species predominating and less than 10% unphosphorylated. In contrast, in failing heart and in myectomy samples from hypertrophic cardiomyopathy patients, the majority is unphosphorylated. Total phosphorylation levels are reduced in failing and myectomy (23 and 39% of normal).

The site-specific antibodies show a distinctive distribution pattern of phosphorylation sites. The 4P band of cMyBP-C contained phosphorylated Ser273, Ser282 and Ser302 but none of these were present in the 1P band, indicating that there must be at least one other site of phosphorylation in cMyBP-C. Phosphorylation at Ser282 was not proportional to the number of sites available. The 2P band contained 302 but not 273 and the 3P band contained 273 but not 302.

P.78

Mutations that alter cTnC Ca^{2+} binding affect interactions with cTnI and cardiomyocyte Ca^{2+} handling

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We have produced a series of cardiac TnC (cTnC) variants with increased (L48Q) or decreased (L57Q or I61Q) Ca^{2+} binding affinity (in solution) that alter Ca^{2+} regulation of force development in skinned myocardium. We are using these reagents to study (1) molecular mechanisms of the thin filament Ca^{2+} signaling pathway, and (2) their influence on intracellular Ca^{2+} metabolism. Solution binding studies indicate increased affinity of the cTnC variants for cTnI in the order L48Q > wild type (WT) > L57Q > I61Q. Molecular dynamic simulation modeling suggests increased Ca^{2+} and cTnI affinity of L48Q vs. WT cTnC results from increased mobility of the B-helix, greater exposure of the hydrophobic patch and stronger interaction of Ca^{2+} with coordinating residues D67, S69 and E76 of site II. Adenoviral overexpression of cTnC variants (2 days) in quiescent adult rat cardiomyocytes resulted in increased shortening rate and magnitude in the order I61Q > L57Q > WT > L48Q. Ca^{2+} transients (Fura-2) were not affected by L48Q, but reduced with L57Q or I61Q cTnC. Cardiomyocytes from infarcted hearts had significantly impaired rate and extent of shortening with an increased Ca^{2+} transient magnitude compared to cardiomyocytes from normal hearts. Both depressed function and Ca^{2+} transients were rescued by L48Q. These data suggest cTnC variants with altered Ca^{2+} binding affinity can influence interaction with cTnI and alter myocardial Ca^{2+} metabolism. HL091368, HL65497 (MR), AHA2310117 (FSK).

P.79

The effect of D175N and E180G mutations of α-tropomyosin on actin-myosin interaction during ATPase cycle

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Hypertrophic cardiomyopathy (HCM)-causing D175N and E180G mutations in α-tropomyosin were examined by incorporation of recombinant α-tropomyosin into reconstituted thin filaments in ghost fibers. To understand the molecular mechanism underlying the disease we labeled Cys190 of wild-type and HCM-mutant α-tropomyosins with the fluorescent probe 5-IAF, and Cys707 of myosin subfragment-1 and Cys374 of actin with 1.5-IAEDANS. The orientation and mobility of probes were studied in the absence or presence of ADP, Na_3VO_4 and ATP by polarized fluorimetry. At different intermediate states of the ATPase cycle, the orientation and mobility of the probe oscillators were shown to change discretely indicating multistep alterations of the conformation of TM, myosin subfragment-1 and actin subdomain 1 during ATP cycle. The E180G mutation strongly modified these changes; in contrast, the D175N had very weak effect on the orientation and mobility of these probes. We suggest that the E180G mutation essentially increases the amplitude of the rotation of the TM strands, SH1 helix and subdomain-1 movements during the ATPase cycle, indicating the increase of the efficiency of work of each cross-bridge. Supported by RFFR (08-04-00960a).

P.80

HGF/c-Met role in postnatal heart development

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Our study aims at exploring the effects of the Hepatocyte Growth Factor (HGF) in cardiac postnatal development, when the transition between the plastic and post-plastic phase occurs. To this purpose, we generated a mouse model with drug-inducible and cardiac-specific

expression of HGF. We performed full genome microarray analysis of mouse hearts at postnatal days P2 and P7. At P7, we found ~200 genes significantly modulated in HGF-expressing vs. control hearts. Several of these genes act in cell cycle regulation, translation, signal transduction, transcriptional regulation, cytoskeleton/extracellular matrix and vesicle traffic. Most of the genes upregulated by HGF were downregulated from day 2 to day 7 in control hearts, suggesting that HGF stimulus opposes at physiological downregulation. Meta-analysis of significantly modulated genes identified a specific network of interactions belonging to the Notch pathway. We confirmed that HGF increases Ki67 expression at P7, indicating that it prolongs the plastic phase in neonatal heart. We also found reduced levels of connexin43 and sarcomeric proteins in newborns, as respect to controls. As adults, mice overexpressing HGF developed a systolic contractile dysfunction, even when HGF expression was suppressed after birth.

These data suggest that HGF/c-Met axis exerts an important role in heart postnatal development and might be implicated in the pathogenesis of cardiac disease with developmental origins.

P.81

The role of myomesin missense mutations on the genesis of hypertrophic cardiomyopathy

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Hypertrophic Cardiomyopathy (HCM) is a cardiac disease characterised by a hypertrophied left ventricle accompanied by a high incidence of sudden cardiac death. We identified a missense mutations in patients with HCM in a gene called myomesin. The N-terminal domain 1 of myomesin interacts with the alpha-helical tail domain of myosin while its C-terminal domain 13 forms antiparallel dimers. To investigate dimerization of Myomesin via domain 13 we performed analytical ultracentrifugation experiments of myomesin domains 11–13. It was shown that the wild type is presented as a stable dimer while the mutation multimerizes at higher concentration. Additionally melting curves experiments of myomesin domains 11–13 revealed that the mutation V1490I unfolds more rapidly than the wild type. We suggest that the myomesin dimers containing the mutation V1490I are more unstable than wild type dimers. Further we transfected neonatal cardiomyocytes with myomesin domains 11–13 wildtype and mutation V1490I. We could find dots attached to the M-Band, which seem to be very specific for Myomesin. The amount of the dots decreases with the expression time. To investigate the development of these dots in the wild type and the mutant we will perform live cell imaging and FRAP experiments.

In the next step Atomic Force Microscopy experiments of wild-type and mutation V1490I will be performed (cooperation with Dr. Irina Agarkova, Zürich). to investigate the elasticity of the mutated myomesin.

P.82

Relevance of protein phosphatase 2A in regulation of myofilament contractility in failing and non-failing human myocardium

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Protein phosphatase (PP) activities are altered in end-stage human heart failure. PP2A activity is reduced in ischemic cardiomyopathy (ICM) compared to non-failing controls. However, it is unknown whether PP2A affects myofilament function in the human heart. Therefore, we investigated the effects of PP2A on myofilament function in failing and non-failing human tissue. Isometric force was measured at different [Ca^{2+}] in single permeabilized cardiomyocytes from ICM, idiopathic dilated cardiomyopathy (DCM) and non-failing (NF) human left ventricular tissue. PP2A incubation (5 U/ml; 40 min) increased Ca^{2+}-sensitivity (pCa50) in NF ($\Delta pCa50 = 0.05 \pm 0.01$) and to a lesser extent in ICM ($\Delta pCa50 = 0.02 \pm 0.01$) and DCM ($\Delta pCa50 = 0.03 \pm 0.01$). No changes were observed in maximal force, passive force and the steepness of the force-pCa relation. The level of phosphorylation of troponin I at protein kinase A sites Ser23/24 was lower in failing compared to non-failing hearts, whereas the overall phosphorylation of other myofilament proteins (myosin binding protein C, desmin, troponin T and myosin light chain 2) determined with ProQ-phosphostaining did not differ. These results indicate that PP2A is involved in the regulation of the myofilament function in non-failing and failing human hearts by increasing Ca^{2+}-sensitivity of force development, possibly through dephosphorylation of troponin I.

P.83

The host muscle environment has got a profound effect on satellite cell function

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Satellite cells are able to regenerate skeletal muscle and repopulate the satellite cell compartment, but their capacity to do this is profoundly influenced by the local host muscle environment. We have used the dystrophin-deficient *mdx* mouse, a model of Duchenne muscular dystrophy, to test the contribution of normal donor satellite cells to regenerated muscle fibres. The muscles of *mdx* mice undergo cycles of degeneration and regeneration, commencing at approximately 3 weeks of age. However, donor cells neither efficiently regenerate myofibres, nor self-renew, when grafted into host *mdx nu/nu* muscles. In an attempt to improve the engraftment efficiency of donor satellite cells, we have modified the host muscle environment in ways that model the changes that occur in dystrophic muscles. These included: high dose of radiation, which incapacitates the majority of resident satellite cells and prevents endogenous muscle regeneration; cryoinjury, which damages locally both muscle fibres and satellite cells but allows muscle regeneration; injection of myotoxins, which cause fibre necrosis, but permit host muscle regeneration. Donor satellite cells grafted into irradiated *mdx nu/nu* host muscles both efficiently regenerated skeletal muscle and self-renewed. However, significantly less donor-derived muscle regeneration occurred in host muscles that had been either cryodamaged or injected with myotoxins. Activation of radiation-resistant host satellite cells did not alter the number of donor-derived myofibres compared to irradiation. These data imply that an empty host satellite cell niche is not an essential requirement for efficient donor satellite cell function.

P.84

Experimental stem cell therapy: role of transplanted bone marrow cells in the skeletal muscle regeneration

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To extend our knowledge of experimental stem cell therapy of skeletal muscle diseases and investigate the role of exogenous adult bone marrow cells (BMCs) in the skeletal muscle regeneration, we intravenously transplanted mouse lacZ+ or GFP+ freshly isolated BMCs into whole-body lethally irradiated immunocompetent mice 7 h after or 4 weeks before the cardiotoxin-induced injury of the recipients' tibialis anterior muscles. Seven to 33 days after the toxin injection, injured muscles were analyzed by X-gal histochemistry or examined for GFP fluorescence. The presence of lacZ gene in injured muscles was determined by qPCR. The skeletal muscles of recipients injured before the transplantation did not regenerate, nevertheless, X-gal positivity was predominantly identified in desmin- and nestin-multinucleated cells resembling giant cells in the injured areas, 14 and 33 days after grafting. On the contrary, the recipients' muscles injured after the transplantation fully regenerated; X-gal or GFP positivity was observed in inflammatory cells 7 days after the muscle injury and rarely, in newly formed myofibers 28 days after the toxin injection. qPCR verified contribution of transplanted BMCs to the population of the lacZ+ cells. Our results confirmed ability of intravenously transplanted exogenous BMCs to settle in the injured skeletal muscle and participate in the skeletal muscle regeneration process.

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P.85

Myogenic properties of human mesenchymal stem cells derived from three different sources

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Mesenchymal stem cells (MSCs) are characterized by their aptitude to differentiate into bone, cartilage and fat. Differentiation into other cell lineages (i.e. skeletal muscle, tendon-ligament, epithelium, neural cells) has been attained with MSCs originated from some tissues. Whether this property is shared by MSC of all sources is not known. To address the question of whether potency of adult human MSCs (hMSCs) is dictated by the tissue of origin, we compared beside their canonical differentiation also the myogenic properties of cells from bone marrow (BM), synovial membrane (SM) and adipose tissue (AT) of 3 donors. hMSCs derived from the three tissues differ in phenotype and differentiation capacity. The proliferative rate of AT derived hMSCs (AT-hMSCs) is higher than that of hMSCs from the other sources. The long-term culture expansion of hMSCs from all tissues is dictated by the donor. Although similar in their in vitro fusogenic capacity, transplanting BM-, SM- and AT-hMSCs previously transduced with a lentiviral vector encoding β-galactosidase into cardiotoxin damaged tibialis anterior muscle (TAM) of NOD/SCID mice revealed at 30 days after treatment highest frequency of hybrid myofibers (HMs) in TAMs treated with AT-hMSCs. The presence of the human myogenic proteins (spectrin and dystrophin) in HMs containing nuclei derived from hMSC argues for myogenic programming of cell of the different origin. Conclusively, the most efficient contribution of AT-hMSCs to myoregeneration together with their highest ex vivo expansion potential and the least demanding procedure for harvesting makes AT-hMSCs the cells of preference for cell-based (gene) therapy for myopathies.

P.86

Adiponectin increases mesenchymal motility in muscle satellite cells

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Adiponectin is an adipocyte-derived hormone with anti-diabetic, anti-inflammatory and anti-atherogenic properties and exerting insulin-sensitizing metabolic effects. The hormone circulates in the plasma as "full-length" and "globular" isoform. Our previous results showed that adiponectin is involved in the regulation and differentiation of mesoangioblasts, a population of non resident muscle progenitors. Our studies are now focused on muscle satellite cells, an acknowledged model of resident stem cells. We found that adiponectin increases the motility of satellite cells towards myotubes as the hormone increases both the migration and the invasion of stem cells. Adiponectin activates the small G-protein Rac1 and induces the expression of Snail and Twist transcription factors, thus allowing stem cells to actively move towards the damage site. Satellite cells preferentially express AdipoR1, the receptor for globular adiponectin, although in response to activation they are autocrine for full-length adiponectin. In keeping, we observed that globular adiponectin is more efficient with respect to the full length form in eliciting a motile response in satellite cells. We are now investigating a possible role of elastase, released in response to pro-inflammatory cues and already reported to generate the active globular isoform, in the regulation of the activity/availability of this new muscle stem cell factor.

P.87

Protein kinase c theta signaling regulates the expression of genes essential for myoblast fusion by regulating focal adhesion kinase activity

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Myoblast fusion is an essential phase of skeletal myogenesis, which occurs during muscle development, as well as during post-natal life for muscle growth, turnover and regeneration. Recently, FAK has been proposed as a potential mediator of myoblast fusion, regulating caveolin-3 and β1D integrin expression. We found that the expression of PKCθ is strongly upregulated following freeze-injury induced muscle regeneration, as well as during in vitro differentiation of satellite cells. Employing both a PKCθ knock out and a muscle specific PKCθ dominant negative mutant mouse models, we observed

delayed body and muscle fiber growth during the first weeks of postnatal life, when compared to WT mice. Moreover, following freeze injury, impaired reorganization of regenerating muscle together with reduced cross-sectional area (CSA) of regenerating, eMyHC (embryonic myosin) positive, fibers was observed. This phenotype was associated to reduced expression of the myogenic differentiation program executor, myogenin, but not of the satellite cell marker, Pax7. Indeed, cultured muscle derived satellite cells from PKCθ mutants, formed thinner myotubes with reduced number of myonuclei and reduced fusion rate, when compared to WT cells. This effect was associated to reduced FAK activation and caveolin-3 and β1D integrin expression. We thus propose that PKCθ signaling is required for cell surface proteins-mediated myoblast fusion, by, at least in part, regulating FAK activity.

P.88

Proliferation of human myoblasts in vitro is enhanced by LPS-induced IL-6 secretion but not by addition of synthetic IL-6

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IL-6 is robustly secreted from the human myoblasts under in vitro conditions. Since it has also been demonstrated that IL-6 stimulate myoblast proliferation, it is assumed that the meaning of this secretion is autocrine and paracrine promotion of the regeneration process which follows muscle injury and starts with myoblast proliferation. However, we found that addition of commercially available synthetic IL-6 had no effect on the in vitro proliferation neither in human myoblasts nor in the C2C12 muscle cell line. It therefore means that IL-6 induced proliferation of muscle regeneration precursors is complex and probably occurs only in the presence of factors which stimulate its endogenous secretion. To test this assumption, we induced the secretion of endogenous IL-6 from human myoblasts by addition of bacterial endotoxin lipopolysaccharide (LPS) to the culture medium. We then collected the medium from these cultures and used it as a conditioned medium in other human myoblast cultures. In comparison to controls grown in the normal medium we found significant increase of BrdU-positive cells after the treatment with conditioned medium. In conclusion, IL-6 alone is not sufficient for the stimulation of myoblast proliferation. Increase of myoblast proliferation after the treatment with conditioned medium taken from LPS-treated myoblasts, highlights the fact that combination of IL-6 with other factors secreted from the myoblasts is necessary for the IL-6 proliferation effect.

P.89

Directed transdifferentiation of fibroblasts to muscle cells by defined factors

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The Duchenne and Becker muscular dystrophies are caused by mutation of dystrophin gene and primarily affect skeletal and cardiac muscle. Cardiac involvement in dystrophic GRMD dogs has been demonstrated by electrocardiographic studies with the onset of a progressive cardiomyopathy similar to DMD patients. In this respect, GRMD is a useful model to explore cardiac and skeletal muscle pathogenesis and for developing new therapeutic protocols. Here we describe the isolation of self-renewing cardiac progenitors from different regions of wild type and GRMD heart. Following appropriate stimulations wt and GRMD progenitors differentiate in smooth muscle cells, endothelial cells, and immature cardiomyocytes albeit with different capacity. In vitro, cardiac GRMD progenitors failed to differentiate into myosin heavy chain/alpha-sarcomeric actinin double positive cells and showed a limited lifespan compared to wild type counterpart. This impairment in terms of proliferation and cardiac differentiation potential could be explained by the continuous regenerative attempts in chronic damage conditions thus exhausting the cardiac progenitor pool. Lineage reprogramming has become a powerful tool to study cell fate choice and represent a promising strategy for cell-based therapy approaches. In this context, we are currently studying a strategy of lineage reprogramming to force transdifferentiation of fibroblast towards muscle cells fate by selective over-expression of master regulator genes.

P.90

Cell therapy of muscular dystrophy with engineered CD133+ cells

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DMD is a genetic disease caused by mutations in dystrophin gene. Forced exclusion (skipping) of a single or multiple exons can restore the reading frame, giving rise to a shorter, but functional dystrophin protein. We selected the GRMD dog, that shares with DMD patient progressive clinical signs and severe myopathy with contractures and premature death. We isolated CD133+ cells from skeletal muscle biopsies of GRMD dogs and we transduced them with lentiviral vectors constructed to convey antisense oligonucleotides able to eliminate the mRNA segment from exon 6 to 8. Under appropriate sedation, the dogs received arterial systemic injections through a catheter introduced in the left femoralis artery and reached the aortic arch at the level of the left subclavia: cells were released mainly in the two large arteries under fluoroscopic guidance in order to provide the whole body musculature. Serial injections of the engineered CD133+ cells do not stimulate an immunoreaction in the treated dogs. The delivery of these cells results in a partial recovery of stiffness and ambulation disability of the treated dogs. The muscle biopsies of the transplanted dogs showed clusters of dystrophin positives fibers. This approach should offer a preclinical evidence for future therapies based on autologous transplantation.

P.91

The effect of hypoxia on the apoptosis in the precursors of human muscle regeneration under in vitro conditions

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Muscle injury is always followed by regeneration starting from the satellite cells located under the basal lamina of adult muscle fibers. Satellite cells turn into proliferating myoblasts which, after fusion into

myotubes, provide new muscle fibers. Myoblasts proliferation is accompanied by apoptosis which reduces the regeneration efficiency. A frequent cause of muscle injury is hypoxia and therefore regeneration often proceeds under hypoxic conditions. Understanding regeneration under such conditions necessitates an insight into the influence of hypoxia on the myoblast apoptosis. Hypoxia induces the expression of different genes whose products are either anti- or pro-apoptotic, depending on the cell type and experimental conditions. Since cellular adaptation to hypoxia is primarily organized by hypoxia inducible factor 1α (HIF-1α)we investigated here the influence of HIF-1α on the apoptotic markers in human myoblasts under hypoxic conditions. Staurosporine-induced apoptosis of normal myoblasts exposed to hypoxia (1% oxygen) which induces the HIF-1α response was compared to apoptosis induced under the same conditions in myoblasts in which HIF-1α had been silenced by siRNA. According to our preliminary results hypoxia reduces the apoptotic markers suggesting HIF-1α mediated anti-apoptotic protection.

P.92

Oxygen concentration does not influence growth and differentiation of clonal population of satellite cells

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Oxygen has been demonstrated to influence proliferation and myotubes differentiation. The mechanism of influence is however mostly unknown. We have recently reported that, satellite cells may be comprised of two distinct populations of cells distinguishable for proliferation and differentiation characteristics. In this study, we focused on cloned and un-cloned murine satellite cells under different oxygen concentrations with the aim of exploring the possible role of oxygen on satellite cells. We examined the growth, differentiation and myogenic genes expression during hypoxia (2% oxygen) and normoxia conditions (20% oxygen) at 5, 10 and 15 days. We demonstrate that despite the same proportion of clones was maintained when mouse satellite cells were cultured at both 2 and 20% O_2, the proliferation rate of un-cloned satellite cells was significantly influenced by oxygen with the growth rate increasing at each time point at the lowest concentration. These data suggest that oxygen tension does not have any influence in both proliferation/differentiation processes in cloned populations. Taken together these results suggest that oxygen concentration may act on a specific subpopulation of satellite cells, which may exert influence on a different one modifying ultimately their proliferation rate.

P.93

Effects of some new single nucleotide polymorphisms (SNPs) in *MRF4* and *PTPRQ* genes on MRF4 expression level in bovine *longissimus dorsi* muscle

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Myogenic regulatory factors (MRFs): Myf5, MRF4, MyoD, MyoG play a key role in skeletal muscle development. Due to the functions that muscle regulatory factors (MRFs) play in muscle growth and development, their genes are considered candidate markers for meat production in farm animals, including cattle. Confirmation of already found and detection of novel polymorphisms is necessary to help researching associations between gene sequence variation and meat production traits in cattle.

Genes *Myf5* and *MRF4* are located on chromosome 5 in cattle and they have the same transcriptional orientation. They are separated by 8.7 kb in mouse, and are linked in all vertebrates. This data suggest that their expression may be controlled by the same regulatory elements. The regulation of linked and functionally related genes has been previously studied in different loci for example β-globin or Hox clusters. Recent studies showed that *cis*-acting elements are generally located between −7.5 and −3.5 kb upstream of *MRF4*. Most of these multiple regulatory elements are located in introns of the *PTPRQ* gene, which is not expressed in skeletal muscle. However, nucleotide sequence polymorphism in *MRF* genes was not thoroughly studied so far.

We detected ten single nucleotide polymorphisms (SNPs) located within the promoter of the *MRF4* gene. Two of these polymorphisms create a haplotype and show influence on the *MRF4* gene expression in bovine *longissimus dorsi* muscle. Two out of six SNPs found in the *PTPRQ* gene show a similar influence on *MRF4* gene expression in the muscle.

P.94

Myosin-activating protein kinases—potential regulators of non-muscle myosin IIB in the heart

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De novo myofibril formation hallmarks cardiomyocyte differentiation during embryogenesis and postnatal cardiomyocyte hypertrophy. Myofibrils are assembled from precursor structures called premyofibrils. One of the main constituents of premyofibrils is non-muscle myosin IIB (NMIIB) that needs to be phosphorylated to form filaments in order to stabilize premyofibrillar structure. In vitro NMIIB could be phosphorylated by smooth muscle/nonmuscle and skeletal myosin light chain kinase (sm/nmMLCK and skMLCK, correspondingly), ILK (integrin- linked kinase), ZIPK (zipper interacting protein kinase) and DAPK (death-associated protein kinase). However, it is not clear which of these protein kinases phosphorylate NMIIB and contribute to premyofibril stabilization at different developmental stages. On the way to solve this problem we demonstrated that sm/nmMLCK, ILK and ZIPK content was higher in adult heart than in fetal heart whereas skMLCK content followed a reciprocal trend. Perhaps, skMLCK is especially important during embryonic heart development. Additionally, we have shown that sm/nmMLCK, skMLCK and DAPK colocalize with NMIIB along myofibril in cultured human fetal cardiomyocytes. In contrast, ILK was found associated with focal contacts in these cells. Based on subcellular localization protein kinases studied we suggest that ILK is less likely to regulate premyofibrillar NMIIB and premyofibril stability whereas other kinases are involved in control of premyofibrils at various developmental stages.

P.95

Myogenic cells delivered through photopolymerizable Hydrogel scaffold improve muscle regeneration

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Regenerative medicine is one of the most exciting approaches in the field of skeletal muscle diseases. In our work we combined delivery myogenic cells with an innovative hyaluronic acid-based Hydrogel. This latter holds great potential for clinical applications, as it is injectable, photopolymerizable in situ with UVA light and forms a non-porous yet permeable scaffold.

In our model, tibialis anterior (TA) muscles of C57BL/6J mice were partially ablated and the pocket was filled with Hydrogel containing either intact muscle fibers, freshly isolated satellite cells or cultured satellite cells derived from C57BL/GFP mice.

We performed hystochemical, immunofluorescence and physiological analyses of the engrafted muscles after 2 and 6 weeks. As control we used muscles engrafted only with Hydrogel and sham operated. TA muscles engrafted with fibers and dissociated satellite cells presented increased muscle mass, significantly higher number of GFP+ fibers and GFP+ satellite cells inserted under the basal lamina of GFP+ and GFP− myofibres when compared to the ones injected with control conditions.

We demonstrated that polymer delivery of myogenic cells is feasible and efficient; further experiments with direct intra muscular injection of Hydrogel are presently ongoing.

P.96

Effects of moderate and high-intensity continuous training and high-intensity interval training on skeletal muscle phenotype and exercise tolerance in heart failure rats

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Exercise intolerance is the pivotal clinical hallmark of heart failure (HF) and it displays a direct relationship with skeletal muscle dysfunction and atrophy. Therefore, aerobic exercise training is an adjuvant therapy for HF that improves skeletal muscle function. However, little is known about the impacts of exercise training regimens based on different intensities in HF, mainly related to changes in skeletal muscle phenotype. Thus, we compared the effects of 8 weeks of aerobic continuous moderate training (CMT, 50–60% of maximal oxygen uptake [VO_2max]) and high-intensity interval training (HIT, 85–90% VO_2max) on skeletal muscle structure (plantaris mass, fiber cross-sectional-area [CSA] and fiber type distribution) and exercise tolerance of myocardial infarction-induced HF rats. HF rats displayed exercise intolerance compared with Sham rats and both CMT and HIT increased VO_2max compared with untrained HF rats. Interestingly, a group of HF rats treated with apocynin also showed higher VO_2max than untreated HF rats, suggesting an important role of NADPH oxidase on exercise intolerance in HF. Both CMT and HIT prevented atrophy by increasing muscle mass and fibers CSA. Indeed, CMT and HIT prevented fiber type shift (type I to IIA) observed in untrained HF rats. Taken together, our results suggest that both exercise training regimens were efficient in preventing skeletal muscle atrophy and fiber type shift in HF rats, which culminated in improved VO_2max and exercise tolerance.

P.97

The effects of treadmill aerobic training with different patterns of load on feed arteries of locomotor and respiratory muscles in the rat

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Since aerobic exercise requires activation of circulation and respiration, aerobic training leads to adaptive changes of muscle fibers and vascular bed, including diaphragm, a principal respiratory muscle. This work was aimed at comparing the effects of constant-rate (CT) and interval (IT) aerobic training on gastrocnemius (GM) and diaphragm (DM) muscles and on reactivity of their feed arteries (GA and DA). Noteworthy, the effects of IT on DA have not been investigated earlier. Male Wistar rats were treadmill trained 6 days/week for 8 week, CT and IT were equalized in total volume of performed work. Comparable increases of maximal oxygen consumption and anaerobic threshold were observed after completion of the CT and IT protocols; the effect of training on oxidative potential in GM was more prominent than in DM. The responses of GA and DA were studied under isometric condition. CT did not affect sensitivity to noradrenaline (NA) in GA, but reduced it in DA. However, IT had no effect on response to NA in either GA or DA. Dilator response of GA to acetylcholine (Ach) increased after CT but not after IT. CT had not effect on DA reactivity to Ach. Along with that we observed, for the first time, the diminished Ach-response in DA after IT; this adverse effect of IT on endothelium needs further investigation. In conclusion, CT and IT exert different effects on muscle arterial bed that may be due to combined effect of systemic and local control mechanisms. Support: RFBR 09-04-01701-a.

P.98

In vivo and in vitro analysis of human skeletal muscle following neuromuscular electrical stimulation

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Neuro-muscular electrical stimulation (NMES) has been used as a tool for muscle strength improvement in athletes and in rehabilitation programs in geriatric, cardiovascular and orthopaedic medicine. NMES has shown to increase muscle mass, strength and exercise capacity (in vivo studies).In this study 14 young (18–35 years of age) healthy, male subjects were subjected to 24, 18-min sessions of isometric (bilateral) NMES of the quadriceps muscle over a period of 8 weeks with three sessions per week. Needle biopsies were taken from the vastus lateralis muscles pre- and post-training. In vivo,

analysis of MVC (maximal voluntary contraction), of the thickness of the vastus lateralis (VL) muscle by ecography, and of muscle fibre conduction velocity and motor unit recruitment by EMG by an array of surface electrodes were performed. In vitro, CSA, specific force, myosin concentration and unloaded shortening velocity of identified types of muscle fibres were determined: 420 fibres (on average 14 fibres per subjects) were analyzed both pre- and post- NMES. MVC contraction and VL thickness increased in all subjects post-NMES compared to pre-NMES of 16 and 14% respectively. Muscle fibre CSA significantly increased post-NMES compared to pre-NMES. MHC isoform distribution shifted in the direction MHC-2X > MHC 2A > MHC-1. The analysis of specific force and unloaded shortening velocity of identified types of muscle fibres and of the results of the electromyographic analysis will be presented at the meeting.

P.99

Different human skeletal muscle architectural adaptations to eccentric and concentric training matched for neural activation

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This study investigated the structural and functional adaptations of human skeletal muscle (vastus lateralis) to pure eccentric and concentric training matched for neural activation. Twelve young healthy males, randomly assigned to a concentric (CG) or eccentric (EG) group, trained on a leg-press machine 3 times/week for 10 weeks with 4 series of 8–10 repetitions with a load of 80% of the concentric or the eccentric 1RM. Maximum voluntary contraction (MVC) torque was measured during isometric contractions from 90° to 150° of knee extension. Muscle volume (VOL) was measured using MRI and muscle architecture (fascicle length, Lf, and pennation angle) was assessed by ultrasonography. After training, the increase in VOL was twofold greater in CG (10%) compared to EG (5%) ($P < 0.01$). Instead, the increase in fascicle length was significantly greater in EG (12%) compared to CG (5%) ($P < 0.01$). Conversely, pennation angle increased more in CG than in EG (30% vs. 5% respectively, $P < 0.01$). PCSA increased by 5% in CG ($P < 0.01$) but decreased in EG, since Lf increased more than VOL. Isometric MVC increased similarly in both groups (CG = 9%; EG = 11%, $P < 0.05$). These findings suggest that the responses to concentric and eccentric training may involve different myogenic and connective tissue-derived mechanisms. This hypothesis seems consistent with observations of different muscle cell signaling responses to concentric and eccentric loading in animal skeletal muscle (Martineau and Gardiner J Appl Physiol 91, 2001; Wretman et al. J Physiol 535, 2001).

P.100

Exhaustive exercise affects contractile properties of single human muscle fibres

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The purpose of the present study was to investigate the effects of long-term exhaustive exercise on the contractile properties of human muscle fibres. Highly trained triathletes ($n = 3$, VO_2max 70 ± 1 ml kg^{-1} min^{-1}) performed a 4 h exhaustive cycling exercise at 75% HRmax, and needle biopsies were obtained in m. vastus lateralis before and after exercise. Whole muscle MHC distribution was $75 \pm 9\%$ (I), $25 \pm 9\%$ (IIa) and $0 \pm 0\%$ (IIx). Fibre bundles were cooled and skinned in a glycerinating solution and stored until analyzed. Single muscle fibre segments ($n = 99$) were isolated and attached to a sensitive force recording transducer, and activated by Ca^{2+} buffered solutions at pH 7.1 to measure mechanically properties and fibre typed by the Sr^{2+} sensitivity. Type1 fibres had a sign. 29% larger CSA than type2 fibres (8600 ± 428 vs. 6662 ± 328 μm^2), and a sign. lower specific force production (P0, 98 ± 4 vs. 165 ± 14 kN/m^2). Following the 4 h exercise, type1 fibres exhibited a sign. 20% decrease in maximum Ca^{2+} activated force (from 0.81 ± 0.04 to 0.65 ± 0.04 mN) and 18% decrease in P0 (from 98 ± 4 to 81 ± 4 kN/m^2). There were no effects of exercise in type2 fibres. Exercise did not affect fibre Ca^{2+} sensitivity (pCa_{50} averaging 5.94 ± 0.01), the threshold for fibre contraction (pCa_{10} averaging 6.18 ± 0.02), nor the force-pCa curve steepness. These data demonstrates that prolonged exercise affects the contractile apparatus in human type1 fibres, which may have a significant impact on muscle function and fatigue.

P.101

Short-term immobilization impairs human single muscle fibre contractility in young and old

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Single muscle fiber contractility is impaired with aging and disuse (D'Antona et al. 2003), but it is not known whether elderly are more severely affected than young by short-term immobilization. In order to investigate this, biopsies were obtained from vastus lateralis in 3 young (Y, 26 years) and 3 old (O, 70 years) healthy men prior to and after 4 days of lower limb immobilization. Muscle samples were stored at −20°C, and at the day of analysis, single fibres were isolated and chemically skinned. Single fibre specific force (SF = maximal Ca^{2+}-activated force/cross-sectional area) were determined. Force-Ca^{2+} relationship were determined in order to assess Ca^{2+} sensitivity (pCa_{50}). Subsequently, fibre types were determined by gel electrophoresis ($n = 132$). Prior to immobilization, SF and pCa_{50} were similar between Y and O in both MHC I and IIa. SF were higher in MHC IIa vs. I ($P < 0.05$) in both Y and O, whereas pCa_{50} did not differ. After immobilization, SF decreased by 27–35% ($P < 0.05$) in Y (MHC I: 95.0 ± 8.0 to 69.7 ± 3.1 kN/m^2; MHC IIa: 139.7 ± 11.0 to 91.1 ± 11.0 kN/m^2) and by 10–13% ($P < 0.05$) in O (MHC I: 78.5 ± 3.0 to 70.7 ± 3.9 kN/m^2; MHC IIa: 160.4 ± 32.2 to 140.0 ± 6.3 kN/m^2). pCa_{50} decreased to a similar extent in Y and O, both in MHC I (5.98 ± 0.03 to 5.83 ± 0.05, $P < 0.05$) and MHC IIa (5.83 ± 0.06 to 5.79 ± 0.04, $P < 0.05$). In conclusion, short-term immobilization impairs human single fibre contractility. Notably, some of the physiological mechanisms causing these impairments seem age-dependent.

P.102

Long-term effects of exercise training and dopamine agonists in patients with uremic restless legs syndrome: a six-month randomized, double blind, placebo-controlled study

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Background: Restless Legs Syndrome (RLS) is common in hemodialysis (HD) patients; however, clinical trials investigating treatment options are very limited. The aim of this study was to compare the effects of a 6-month intervention with dopamine agonists or intradialytic exercise training on physical and mental parameters as well as on RLS symptoms in HD patients. Methods: Randomized double blind controlled design. 32 HD patients with RLS were randomly assigned into: (1) exercise training group ($N = 16$, 56.4 ±12.5 years), (2) dopamine agonists group (ropinirole 0.25 mg/day) ($N = 8$, 55.7 ±10.4 years) and (3) placebo group ($N = 8$, 56.8 ±16.5 years). RLS severity was assessed with the IRLS severity scale, physical performance by a battery of tests, muscle size and composition by CT, body composition by DEXA, while depression levels, sleep quality, daily sleepiness and quality of life (QoL) were assessed with validated questionnaires. Results: Exercise and dopamine agonists were both effective in reducing RLS symptoms by 46 and 54% respectively with no adverse effects. Both regimes significantly improved QoL, however, only the dopamine agonists group improved sleep quality while only exercise training increased lean body mass (LBM) and reduced fat infiltration resulting a significant increase in their physical performance. No changes were observed in the placebo group apart of the depression score which deteriorated significantly after the 6 month intervention. Conclusion: Low dosage dopamine agonist treatment was as effective as chronic exercise training in reducing RLS symptoms and improving aspects of the quality of life in HD patients. However, only exercise increased physical performance and improved muscle and body composition parameters; such changes could be translated into improved survival.

P.103

Muscle damage induced by an acute bout of plyometric exercise

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Plyometrics refer to exercise that exploits the stretch–shortening cycle, which starts with a rapid stretch of a muscle (eccentric phase), followed by rapid shortening of the same muscle (concentric phase) and an increase in the force and power output of the activated muscles. Only indirect evidence suggests that plyometric exercise mainly affects the fast-twitch muscle fibers, although none of these studies provided morphological results to confirm this. The purpose of this study was to determine what kind of damage is induced by an acute bout of plyometric exercise and which type of muscle fibers are predominantly injured. Ten healthy sedentary male subjects performed ten sets of ten maximal vertical jumps, separated by a 60 s recovery time between sets. A baseline biopsy was obtained from the vastus lateralis muscle of each subject from a random leg nine days before the exercise protocol was performed, while a second biopsy was obtained on the third day following the plyometric exercise intervention on the leg which was not biopsied the first time. Muscle samples were analyzed with light and electron transmission microscopy. Blood samples were drawn immediately before and after (6 h, 1, 2, 3, 4 days) the plyometric exercise intervention and they were analyzed for CK, Mb, and LDH concentration. The acute bout of plyometric exercise preferentially damaged FOG and FG fibers (type II fibers) inducing sarcomere Z-line streaming observably only throughout transmission electron microscopy.

P.104

RealtimePCR investigations of MyHCs and myogenic factors in muscle remodeling induced by electrostimulation in young and elderly subjects

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This study describes the molecular mechanisms involved in muscle response to neuromuscular electrical stimulation (NMES). NMES protocols exerts significant effects on the muscle phenotype and function. The comparison between before and after NMES treatments may indicate the functional changes of muscle and the molecular basis of muscle remodeling. Total mRNA of Vastus lateralis muscle of young versus elderly subjects, both treated with NMES, were extracted and retrotranscribed. The expression pattern of skeletal muscle myosin heavy chains (MyHC 1, 2A and 2X) and myogenic factors (IGF1, MURF1, Myf5, Pax7 and MSTN) were analyzed by means of a Real-Time PCR approach. The NMES treatment causes consequences in both young and elderly subjects although more emphasized in young. Our results indicated high variability of response in terms of gene expression probably due to basal level of physical activity of subjects. Proteomic and in vivo functional studies performed in collaboration with other research groups will help to understand the real effects of NMES on muscle plasticity during aging.

P.105

Subcellular localization-dependent usage of skeletal muscle glycogen during 1 h exhaustive arm and leg exercise

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In the present study, transmission electron microscopy was used to estimate the subcellular localization of glycogen in the leg (*m. vastus lateralis*) and arm muscles (*m. triceps brachii*) of 10 elite cross country skiers pre and post a 1 h cross country skiing time trial. Three subcellular localizations were defined: intramyofibrillar (Intra), intermyofibrillar (IMF) and subsarcolemmal (SS).

In the arms, the decrease in glycogen content following the time trial was dependent on subcellular localization ($P < 0.0001$), where Intra glycogen decreased to lower values (median and inter quartile range) compared with IMF and SS glycogen (10% (6:26) of pre value versus 21% (15:49) ($P < 0.001$) and 20% (11:57) ($P < 0.001$) of pre values, respectively). In the legs, no difference was found between the different localizations, where glycogen decreased to 67% (29:119), 63% (36:97) and 57% (31:97) of pre values for Intra, IMF and SS glycogen, respectively ($P = 0.54$).

For arm and leg together, these findings indicate that glycogen usage depends on subcellular localization when glycogen levels become very low (arms), but not when only a modest amount of glycogen is used (legs). This suggests that at the end of glycogen-depleting exercise, IMF and SS glycogen are retained, while Intra glycogen is further depleted. Thus, the question emerges as whether low Intra glycogen content is the signal to cease forceful muscle contractions, i.e. muscle fatigue during prolonged exhaustive exercise?

P.106

MGF an isoform of the IGF-I induces human myotube hypertrophy and activates human muscle satellite cells

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Skeletal muscle is a postmitotic tissue and a population of myogenic precursors called satellite cells are responsible for its growth and repair. Mechanically challenged muscle produces a growth/repair factor, called MGF (Mechano Growth Factor). The MGF has a unique E domain resulting from a 49 bp insert in the human, Ec, (52 bp in rodent; Eb), during the IGF-I gene splicing. Studies have shown that MGF protects against oxygen free radical damage and has special functions in damage, repair and adaptation. It has been shown that age-related sarcopenia is related with a reduced expression of MGF. We have studied the effects of MGF peptide on proliferation and differentiation of human primary muscle cell cultures isolated from healthy subjects of different age groups. We found that the MGF peptide significantly increased the proliferative life span of satellite cells from neonatal and young adult but not from old adult muscle. In differentiated cultures, myotube size was assessed by counting the number of nuclei per myotube. A hypertrophic morphology was observed with the MGF treatment of satellite cells from all three age groups. Interestingly, the mean number of nuclei per myotube was higher in cultures from older subjects. Myoblasts, defined by the expression of desmin, do not all fuse and are called reserve cells. A significant decrease in the percentage of reserve cells correlated with an increase in the number of nuclei in the myotubes was observed when MGF was added to cultures from old muscle. These data indicate that MGF could be a rate limiting factor in muscle repair and adaptation in the elderly. It was also found that the MGF peptide even at low concentrations induced proliferation and fusion and was more effective than full length IGF-I. Thus, it is concluded that the unique C terminal peptide of only 24aa of the MGF isoform of IGF-I has a marked ability to induce the satellite cell activation for muscle repair and maintenance.

P.107

DHT modulates force production and amino acid uptake in mammalian fast- and slow-twitch skeletal muscle fibres via an EGFR dependent pathway

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Anabolic–androgenic steroids (AASs) such as testosterone are commonly prescribed for the treatment of sarcopenia and cachexia because of their body building properties. These effects are mediated via the androgen receptor and involve gene transcription and mRNA translation. Therefore, they take several days to be manifested (Beato M. Cell 56:335–344, 1996). In addition, AAS also have non-genomic actions whose cellular-signal transduction events and physiological functions are poorly understood (Heinlein CA and Chang C. Mol Endocrinol 16:2181–2187, 2002). In this study, we investigated the effects of treating small muscle fibre bundles isolated from the extensor digitorum longus (edl; a fast-twitch muscle) and soleus (a slow-twitch muscle) of adult female mice with testosterone (T) and dihydrotestosterone (DHT). Our results show that treating the muscle fibres bundles with physiological concentrations of DHT, and not T, increases the phosphorylation of ERK 1/2 in both fibre types and the 20 kDa regulatory myosin light chains in the fast-twitch fibres only. These effects were accompanied by an increase in force production and the uptake of isoleucine in the fast-twitch fibres only. Moreover, all these effects were abolished by the EGFR and MEK inhibitors, tyrphostin 1478 and PD98059, respectively; suggesting that they were mediated through the EGFR and involve the activation of the MAPK pathway (Hamdi MM and Mutungi G. J Physiol 588:511–525, 2010). From these results we suggest that; (1) DHT is the more potent anabolic hormone in mammalian skeletal muscles. (2) Its acute actions prepare the muscle for its genomic actions.

P.108

Myonuclear domain size and 3D myonuclear organization in single muscle fibers from myostatin deficient or IGF1 overexpressing mice

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Myostatin deprived or IGF-1 over-expressing mice are characterized by a 2–3-fold increase in muscle size compared to controls. Despite the hypertrophy these mice show significant difference in force generating capacity, i.e., maximum force normalized to muscle fiber cross-sectional area or specific force. That is, specific tension in IGF1 overexpressing transgenic mice is similar to controls while significantly lower in the myostatin knock out mice. The mechanism underlying this compromised muscle function is unknown. In an attempt to explore this mechanism we have investigated the size of cytoplasmic volume (myonuclear domain MND) supported by individual myonuclei in single muscle fiber segments from myostatin deficient, IGF1 over-expressing and control mice, using a novel algorithm to measure the MND in 3D. Single skinned muscle fiber

segments were mounted at fixed sarcomere length corresponding to optimum filament overlap for force generation and stained with DAPI (myonuclei) and rhodamine (actin). Our image analysis algorithm was highly effective in determining the spatial organization of myonuclei and distribution of MNDs along the length of the fiber. Early results point towards an inverse relationship between MND and specific force. This implies that hypertrophy is primarily due to expansion of existing MNDs in myostatin knock-outs, and addition of more myonuclei in IGF1 over-expressing mice. This is suggested to have significant effects on transcriptional control of protein synthesis/degradation, turnover rates and/or posttranslational modifications of contractile proteins. We conclude that a maintained MND size is a prerequisite for force generation capacity in hypertrophied muscle fibers.

P.109

Local effects of intramuscular mechano growth factor administration on intact rats and during the recovery from alcoholic myopathy

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Human recombinant MGF (obtained from A.N. Bakh Institute of Biochemistry, RAS) was expressed in *Saccharomyces cerevisiae* to the level of 50 mg/l of physiologically functional product in the culture medium. Chronic alcoholic myopathy is known to produce pronounced atrophy, apoptotic events and suppress muscle satellite cell proliferation. We induced alcoholic myopathy in rats by prolonged alcohol intake (mean 25 g ethanol/kg/day for 16 weeks). The recovery was performed for 4 weeks and was accompanied with MGF administration (IM in medial gastrocnemius muscle in a dose of 0.015 mg per 100 μl of physiological saline for each muscle). In alcoholic myopathy group prolonged MGF application didn't increase myofiber CSA, caused 20% increase in BrdU incorporation, restore of myonuclear number, had no effect on M-cadherin synthesis and 50% decreased Pax-7 expression as compared to control group. MGF was also applied once in intact rats by IM injection in m. medial gastrocnemius in a dose of 0.015 or 0.030 mg per 100 μl of physiological saline per each muscle. The pure saline was applied to control rats. Proliferation intensity (BrdU incorporation), Pax-7 and M-cadherin expression by satellite cells were assessed 1, 2 or 5 day after the injection. We suggest that further fate of satellite cells, proliferated by the influence of MGF was the fusion with myofiber or myogenic differentiation.

P.110

The role of G-CSF in the growth and development of skeletal muscle cells in vitro

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Granulocyte-Colony Stimulating Factor (G-CSF) is a cytokine which stimulates the production of hematopoietic stem cells from bone marrow. Since its discovery and approval for clinical use, various roles for G-CSF outside the hematopoietic system have emerged. Recently, G-CSF treatment has been shown to increase skeletal muscle mass, strength and regeneration in rodent models of muscle disease and damage. However, the molecular mechanisms underlining these responses are poorly understood. In cells expressing the G-CSF Receptor (G-CSFR), ligand binding activates several intracellular signalling cascades such as JAK/STAT, Akt, and MAPK/ERK. These signalling pathways are of vital importance in the regulation of skeletal muscle during hypertrophy, atrophy and regeneration. However, it is unknown whether the G-CSFR is expressed in skeletal muscle, or if these signalling pathways are activated in response to G-CSF treatment. In the present study, the expression of the G-CSFR was detected in C2C12 and human primary muscle cells. G-CSF treatment in C2C12 myotubes increased the phosphorylation of STAT3 and ERK1/2, two proteins implicated in skeletal muscle growth and differentiation. Further studies will determine if G-CSF is involved in the regulation of proliferation and differentiation in these cells. Preliminary data shows Akt phosphorylation is increased, a known regulator of protein synthesis and degradation. However, protein synthesis and degradation remain unchanged.

P.111

The role of sepsis in the development of limb muscle weakness in a porcine ICU model

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In critically ill ICU patients, the mechanisms underlying the limb muscle weakness termed acute quadriplegic myopathy remain unclear. Mechanical ventilation and immobilization-induced modifications may play an important role, but sepsis may also be involved. The aim of the present study was to examine how sepsis aggravates ventilator and immobilization-related limb dysfunction. Hence, biceps femoris structure, function and gene expression were investigated in piglets in response to a combination of mechanical ventilation and endotoxin-induced sepsis for 5 days (SEP) and compared with mechanically ventilated animals for the same duration (MECH) and a sham-operated control group (CTL). Results highlighted that on day 5, muscle fiber size did not differ between SEP, MECH and CTL groups. However, a significant decrease in single fiber maximal force normalized to cross-sectional area (specific force) was observed in the SEP group when compared with the other two groups. In addition, microarray data showed a dysregulation of more than 500 genes, such as an increased expression of genes involved in chemokine activity, innate immunity and apoptosis. A decreased expression in genes regulating heat shock proteins was also apparent. Therefore, it appears that sepsis has an additive deleterious role in acute quadriplegic myopathy. Sepsis-induced molecular mechanisms involving heat shock proteins and chemokine/innate immunity are forwarded as probable mechanisms underlying the decreased force generating capacity (specific force).

P.112

Translational suppressions of atrophic regulators by a microRNA integrate resistance against skeletal muscle atrophy

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Muscle atrophy occurs in many pathological states and results primarily from accelerated protein degradation by the ubiquitin–proteasome pathway. We used dexamethasone to induce muscle wasting and investigated the role of a microRNA (miRNA) in the control of muscle-specific E3 ubiquitin ligase MAFbx/atrogin-1 as well as MuRF1. Here we report for the first time, a single miRNA suppresses the key regulators in the muscle atrophy program and the integrated suppression results significant muscle phenotype. We first show that miR-23a suppresses the translation of both MAFbx/atrogin-1 and MuRF1 by binding to the 3′ UTR of the mRNAs. In parallel, ectopic expression of miR-23a is sufficient to protect myocytes from atrophy in vitro and in vivo. We also subjected miR-23a transgenic mice to dexamethasone treatment and the dexamethasone-induced muscle atrophy was attenuated in these mice. It was recently demonstrated that a single miRNA mildly affects production of hundreds of proteins, however our discovery suggests that even a single miRNA can lead significant phenotypes to repress multiple regulators in adult tissues.

P.113

Involvement of autophagy in the pathogenesis of cancer-induced muscle wasting

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Background/aims: Skeletal muscle atrophy associated with chronic diseases results from the degradation of myofibrillar proteins that eventually depends on the ubiquitin–proteasome system. Recently, the possible contribution of the autophagic/lysosomal pathway has been proposed. The present study has been aimed to evaluate whether autophagy plays a role in cancer-induced muscle wasting.
Methods: Balb-c mice were divided into controls and tumor bearers (TB), the latter receiving 5 × 105 C26 cells. After 11 (10% weight loss) or 14 (30% weight loss) days mice were sacrificed, the gastrocnemius excised and stored for further analysis. Levels of Beclin1, LC3B-II, Cathepsin L, p62 and NBR1, accepted markers of autophagy or lysosomal proteolysis, were determined by Real-Time PCR and/or western blotting. Cathepsin B + L enzymatic activity was analyzed in vitro measuring the cleavage of fluorogenic substrates.
Results: At day 14 of tumor growth, TB showed a decrease in muscle mass and fiber cross sectional area, associated with an increase in Beclin1, LC3B, Cathepsin L, p62 and NBR1 mRNA levels. Beclin1, LC3B-II and p62 protein expression increased after both 11 and 14 days of tumor growth. Interestingly, cathepsin B + L enzymatic activity was not altered in TB at day 11, while decreased at day 14.
Conclusions: Muscle wasting in TB is associated with the accumulation of autophagosomes that ultimately leads to the saturation of the autophagic machinery. Further analysis will be useful to understand if autophagy blockade can prevent cancer-induced muscle atrophy.

P.114

The Haptoglobin deficient mouse as a novel model to study the effects of oxidative stress in the muscle

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Background: Oxidative stress is a relevant cause of sarcopenia and a genetic model to analyze the underlying mechanisms of this process is missing. Haptoglobin (Hp) removes free Hb, thus contributing to the prevention of the oxidative damage. During hemolysis Hp deficient mice show an enhanced renal oxidative damage
Aim: To study the morphological, functional and molecular modifications undergone by skeletal muscle upon Hp deficiency.
Materials and Methods: 5 months old male $Hp^{-/-}$ and control ($Hp^{+/+}$) mice were used to evaluate: cross-sectional area (CSA) of muscle fibers, the abundance of Atrogin-1 and MuRF1 (by real time PCR). Grip test was employed before and after 3 h of rotarod exercise (21 rpm) to assess the effects of fatigue on muscular strength.
Results: $Hp^{-/-}$ mice showed: increased expression of genes involved in muscle atrophy including Atrogin1 and MuRF1 and decreased (by 10%) tibialis CSA as compared to controls. Muscular strength, which was similar in basal conditions, was significantly decreased in $Hp^{-/-}$, but not in controls following a prolonged exercise.
Conclusions: Hp deficiency impacts on muscular fiber size and function. The upregulated expression of the atrogenes found in the muscle of $Hp^{-/-}$ mice is consistent with the activation of a proteolytic pathway, likely due to the oxidative stress undergone by this model. The $Hp^{-/-}$ mouse can then be considered as a novel model to investigate the cause/consequences of oxidative stress on muscular phenotype.

P.115

The anterior cruciate ligament rupture increases the atrogenes expression in quadriceps muscle of rats

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Neurophysiological mechanisms have been proposed to explain the atrophy and weakness of quadriceps after anterior cruciate ligament (ACL) rupture, but there is no description about molecular pathways in that situation. We hypothesized that ubiquitin proteossome pathway is involved due to atrogin-1 and MuRF1 expression. Thus, we evaluated the mRNA expression of atrogin-1 and MuRF1, and the cross sectional area (CSA) of vastus medialis (VM), rectus femoris (RF) and vastus lateralis (VL) muscles. Wistar rats were evaluated after 24 and 72 h of ACL rupture, and divided as follows: Control (C24; C72), Sham (S24; S72—Surgery without ACL rupture) and

ACL (ACL24, ACL72—ACL ruptured). After 72 h, muscle weight/body weight index decreased around 14% for VM and VL muscles in S72 and ACL72, while RF only in ACL72. Muscle fiber CSA of VM decreased in both S72 (28, 91%) and ACL72 (24%), and of VL reduced in ACL72 (22%) compared to control values ($P < 0.05$). The MuRF1 and atrogin-1 expressions increased in all muscles and periods investigated compared to control ($P < 0,05$) presenting the highest levels in 24 h for VM (MuRF1: S24-459%, ACL24-989%; atrogin-1: S24-796%, ACL24-1135%), and in 72 h for both VL (MuRF1: S72-2057%, ACL72- 3123%; atrogin-1: S72-1570%, ACL72-3169%) and RF (MuRF1: S72-166%, ACL72-367%; atrogin-1: S72-1154%, ACL72-1482%). These results showed that either the ACL rupture or surgery procedure activate ubiquitin proteossome pathway in quadriceps muscle of rats.

P.116

Oxidative stress, apoptosis and proteolysis in skeletal muscle repair after unloading

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Although several lines of evidence link oxidants and inflammation to skeletal muscle wasting via regulation of apoptosis and proteolysis, little information is available on muscle repair. The present work was designed to study oxidative stress, inflammatory cytokines, apoptotic or proteolytic pathways during the early (1 and 5 days) and later (14 days) stages of the regrowth process subsequent to 14 days of hindlimb unloading. During the early stages, muscle mass recovery (day 5) was facilitated by downregulation (day 1) of pathways involved in muscle proteolysis (μ-calpain, atrogin-1 and muscle ring finger-1 mRNA) and upregulation of Beclin-1 (day 5). At the same time, oxidative stress remained still enhanced whereas the increased uncoupling protein 3 mRNA recovered. Increased caspase-9 (mitochondrial-driven apoptosis) and decreased caspase-12 (sarcoplasmic reticulum-mediated apoptosis) activation was also normalized at early stages (day 5). Conversely, the receptor-mediated apoptotic pathway initiated by tumor necrosis factor-α (TNF-)binding and promoting the activation of caspase-8, remained elevated till 14 days. Our data suggest that at early stages, muscle repair is mediated via the modulation of mitochondrial-driven apoptosis and muscle proteolysis. Despite full muscle mass recovery, oxidative stress and TNF-α-mediated apoptotic pathway are still activated till later stages of muscle remodeling.

P.117

Electrostimulation during hindlimb unloading modulates PI3K-AKT downstream targets without preventing soleus atrophy and restores slow phenotype through ERK

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Our aim was to analyze the role of PI3K-AKT and MAPK signaling pathways in the regulation of muscle mass and slow-to-fast phenotype transition during hindlimb unloading (HU). For that purpose, we studied, in rat slow soleus and fast EDL muscles, the time-course of anabolic PI3K-AKT-mTOR, catabolic PI3K-AKT-FOXO and MAPK signaling pathways activation after 7, 14, and 28 days of HU. Moreover, we performed chronic low frequency soleus electrostimulation during HU in order to maintain exclusively phenotype and so to determine more precisely role of these signaling pathways in the modulation of muscle mass. HU induced a down-regulation of the anabolic AKT, mTOR, p70S6K, 4EBP1 and GSK3β targets and an up-regulation of the catabolic FOXO1 and MURF1 targets correlated with soleus muscle atrophy. Unexpectedly, soleus electrostimulation maintained p70S6K, 4E-BP1, FOXO1 and MURF1 to control levels, but failed to reduce muscle atrophy. HU decreased ERK phosphorylation while electrostimulation enabled to maintain ERK phosphorylation similar to control level. Moreover, slow-to-fast myosin heavy chain phenotype transition and up-regulated glycolytic metabolism were prevented by soleus electrostimulation during HU. Taken together, our data demonstrated that the processes responsible for gradual disuse muscle plasticity involved PI3-AKT and MAPK pathways in the imbalance between protein synthesis and degradation, and the regulation of contractile phenotype and metabolic profile. However, electrostimulation during HU restored PI3K-AKT and ERK pathways activation without counteracting the decrease in mass suggesting the implication of other signaling pathways in disuse muscle plasticity.

P.118

Postmortem degradation of the skeletal muscle organ in pigs at two different storage temperatures. A histological, fine structural and histochemical approach

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Skeletal muscle comprises the largest homogenous compartment of the body and it's true to life state is well known. In contrast, knowledge on *postmortem* changes of histological and fine structural features of the skeletal muscle organ is scarce and relates primarily to meat quality studies on mammals such as cattle and sheep. To our knowledge, with regard to specific muscle fibre types no studies have been published to date distinguishing between the *postmortem* degradation patterns of the varying muscle fibre types. The purpose of this study is to analyse the extra- and intracellular structural time dependent *postmortem* changes of skeletal muscle as well as to characterise specific degradation patterns of the different muscle fibre types in the *M. biceps femoris* of the pig over a period of 21 days *postmortem* at two different storage temperatures (4 and 22°C). Transversally as well as longitudinally sectioned samples are analysed for changes of skeletal muscle (fibre type specifically), connective and adipose tissues as well as vascularisation both on the light- and electron microscopical level. Furthermore a histochemical analysis is carried out regarding specific fibre type distribution. First results show obviously systematically time-and temperature dependent histological and fine structural changes of the different tissues and cells of the skeletal muscle organ. Moreover, there is clear evidence for a muscle fibre type specific degradation.

P.119

Effects of mutated SOD1 overexpression in muscle cells

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The Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by a progressive loss of motoneurons. About 10% of cases are inherited (fALS) and one-fifth of these cases are linked to mutations in the gene coding for the Cu/Zn superoxide dismutase type 1 (SOD1). Many studies suggest that SOD1 toxicity is non-cell autonomous, involving cells connected to motoneurons and muscle might be a target of toxicity. Mutant SOD1 expression in motoneuronal cells (NSC34) resulted in nuclear exclusion, formation of insoluble species and proteasome impairment, while in muscle cells (C2C12) no insoluble species were formed and mutated SOD1 clearance was increased. By using YFPu (a proteasome reporter protein) we found that mutant SOD1 doesn't alter proteasome activity, suggesting that the degradative system of muscle cells is more efficient than that of motorneuron. We utilized C2C12 cells and gastrocnemius muscles of SOD1 mice to evaluate the effect of mutant SOD1 on the gene expression of proteins involved in muscle pathophysiology, as: MyoD, myogenin, atrogin-1, TGFβ1. The results show that mutant SOD1 up-regulates the expression of these genes both in C2C12 and skeletal muscle; in the latter case, alterations occur at the pre-symptomatic stage preceding motoneuron loss, indicating that these modifications can be primary, rather than secondary, to disease onset and progression. Grants: Telethon, Italian Ministry of Labour, Health and Social Affairs, Fondazione Cariplo.

P.120

Time-course changes in ubiquitin ligases expression during unloading, disuse combined with stretch, reloading in normoxia and under low oxygen

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We aimed at studying of the time-course of the ubiquitin ligases MuRF-1, MuRF-2, and MAFbx during functional unloading and disuse combined with stretch (3, 7, and 14 days), and during the first week of reloading of the atrophied by disuse soleus. We also were interested in the effect of low oxygen consumption on the ubiquitin E3 ligases expression level at the early stage of reloading after disuse. We measured the dry weight of the muscle, reflecting the total protein content. Expression of all the ligases markedly increased at the 3rd day of unloading, and remained high till the 14th day. At the 3rd day of reloading their expression rate also was increased, and declined to the control to the 7th day. Stretch during unloading caused significant decrease in MAFbx expression rate since the 3rd day, while MuRF-1 and 2 expression increased at the 3rd day and declined to the 7th day of HS with stretch. Thus 3 days of stretch combined with HS are the period of significant load, and only after a week stretch leads to the proteolysis/synthesis balance shift towards less intensive proteolysis. Moderate normobaric hypoxia allowed decreasing of the MuRF-1 and MAFbx at the 3rd day of reloading, leading to the total protein content increase even higher than in the control, while MuRF-2 mRNA level decreased only to the 7th day.

P.121

Rolipram, a selective phosphodiesterase 4 inhibitor, reduces muscle protein catabolism by inhibiting atrophy/autophagy-related genes expression through cAMP pathway

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Although it is well known that phosphodiesterase (PDE) inhibitors are effective at attenuating muscle atrophy in rodents, the molecular underlying mechanism remains unclear. The present study was undertaken to investigate the in situ and in vitro effect of rolipram, a selective inhibitor of PDE4 (the major isoform expressed in skeletal muscle), on regulating muscle proteolysis. Microdialysis experiments in anaesthetized rats showed that in situ perfusion of skeletal muscle with rolipram (10-3 M) increased muscle blood flow and reduced by 70% ($P < 0.05$) the interstitial (I)-arterial (A) tyrosine concentration difference, indicating a reduction in muscle protein catabolism. The addition of rolipram (10-5-10-3 M) to the incubation medium increased muscle cAMP levels and decreased overall proteolysis in a dose-dependent manner in both soleus and EDL muscles of fed animals. In EDL, the highest concentration of rolipram (10-3 M) decreased the basal activity of ubiquitin–proteasome system (50%) as well as the expression of GABARAP (70%) and atrogin-1 (55%) independently of Akt Ser 473 phosphorylation. Furthermore, rolipram suppressed the 2-days fasting induced expression of LC3, GABARAP, atrogin-1 and MuRF-1. These findings suggest that the antiproteolytic effects of rolipram are mediated through the activation of cAMP signaling which leads to the suppression of autophagic/lysosomal and proteasomal pathways and atrophy-specific gene transcription.

P.122

Effects of leucine supplementation under muscle atrophy occur via UPS system and independently of protein synthesis

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The aim of this study was to assess the effect of leucine supplementation on elements of the ubiquitin–proteasome system (UPS) in skeletal muscle during immobilization, by submitting rats to a leucine supplementation protocol during hindlimb immobilization up to 7 days. We determined muscle mass; cross-sectional area (CSA); gene expression of E3 ligases/deubiquitinating enzymes; content of ubiquitinated proteins; and rate of protein synthesis. Our results show that leucine supplementation markedly attenuates the decrease in the CSA in soleus muscle type I fibers ($\sim$20%), induced by immobilization. Interestingly type II fibers under disuse did not benefit from leucine supplementation. Leucine supplementation severely minimized ($\sim$50%) the early transient boost in MuRF1 and MAFbx/atrogin-1 gene expression observed after 3 days of immobilization. Western blot for poly-ubiquitinated proteins shows a decrease in the content of ubiquitinated protein in leucine supplemented animals, which is paralleled with the reduction in E3 ligases gene expression. As expected, protein synthesis rate decreased by immobilization ($\sim$40%) and was not affected by leucine supplementation. Our results strongly suggest that leucine supplementation attenuates muscle wasting induced by immobilization via minimizing gene expression of E3 ligases, which consequently could downregulate UPS-driven protein degradation. Financial Support: FAPESP, CNPq.

P.123

Some parameters of the protein synthesis regulation during chronic alcohol intoxication in human

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Translation regulation in the muscle atrophied by the prolonged or acute alcohol intoxication is important for clinical and fundamental research. Some data on the translation regulation were obtained using animal model, while protein synthesis regulation in human is poorly understood. We examined 15 patients with alcohol induced atrophy (with or without myofiber atrophy type II + I and only type II) and 3 volunteers (control). Concentration of IGF-I in serum, phosphorylation level and content of the ribosome kinases (p70 s6k and p90 RSK) in vastus lateralis were measured. Serum level of IGF-I was significantly decreased in all patients groups in contrast to the control level. Quantity and phosphorylation level of p70 s6 kinase were significantly decreased in the group with type I + II myofiber atrophy, whereas in the other groups wide variations were observed. The joint results of 3 groups of patients significantly differed from control. We did not observe any difference in p90 RSK phosphorylation level and total content, as compared with control. Thus, chronic alcohol consumption caused significant decrease in serum IGF-I level and p70 s6 kinase activity, whereas activity of p90 RSK remained unchanged. The data obtained are of importance, since both ribososme kinases are the downstream of IGF-1-dependent pathway. The study was supported by the program "Fundamental science and medicine", RAS.

P.124

Acute cold exposure increases proteolysis but does not change the rates of protein synthesis in skeletal muscles of rats

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It has been shown that sympathetic nervous system exerts anabolic actions on muscle protein metabolism by inhibiting proteolysis and stimulating protein synthesis. To better understand the physiological role of catecholamines on skeletal muscle protein metabolism, we investigated the different proteolytic activities and the rate of protein synthesis in an in vivo condition of continuous sympathetic activation.

Male Wistar rats ($\sim$80 g) were exposed to cold (4°C) and compared to controls (25°C). After 24 h, the animals were killed and soleus and extensor digitorum longus (EDL) muscles were removed to measure the rates of protein synthesis and degradation. Rates of overall proteolysis (nmol of tyrosine/mg^2 h) of rats exposed to cold were higher than controls in both muscles: soleus (0.637 $\pm$ 0.032 vs. 0.477 $\pm$ 0.011) and in EDL (0.423 $\pm$ 0.028 vs. 0.310 $\pm$ 0.019). The activity of ubiquitin–proteasome system was increased in soleus (48%) as well as in EDL (61%) by cold. The activity of calcium dependent system was markedly increased only in EDL (319%). No differences were observed in lisosomal proteolysis and in the rates of protein synthesis, either in soleus or in EDL of rats at 4°C compared to controls.

Ubiquitin–proteasome and calcium dependent systems are responsible for the increased overall proteolysis in animals exposed to 24-h cold. In parallel with catecholamines, corticosterone and insulin may play an important role in these responses induced by cold.

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P.125

Creatine supplementation does not attenuate dexamethasone-induced skeletal muscle atrophy and impaired glucose homeostasis in rats

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Aim: To test whether high-dose creatine (CR) supplementation would prevent of high-dose dexamethasone (DEXA)-induced skeletal muscle atrophy and maintain glucose homeostasis in rats.
Methods: Adult male Wistar rats (400–415 g) were randomly assigned into DEXA (DEX), control pair-fed (CON-PF), DEX-A + CR (DEX-CR), and CR pair-fed (CR-PF)-treated groups. Animals received 5 mg kg^{-1} of DEXA and 2% of body weight of CR per day during 7 days through drinking water.
Results: After 7 days of DEXA treatment, CR supplementation did not attenuate soleus, plantaris, and EDL muscle atrophy, but CON-PF significantly increased soleus mass. DEXA treatment led to a reduced phospho-Ser473-Akt protein levels which was potentiated by CR supplementation. In addition, DEXA and DEXA-CR treatments also reduced phospho-Ser253-FoxO3a protein levels and increased MuRF-1 protein levels in plantaris. Even though total GLUT-4 protein levels in plantaris were similar among groups, DEXA and DEXA-CR treatments significantly decreased membrane GLUT-4 expression, which culminated in elevated blood glucose level in DEXA and DEXA-CR.
Conclusion: Altogether our results provide evidence for DEXA-induced skeletal muscle atrophy and impaired glucose homeostasis. Of interest, high-dose CR supplementation did not prevent DEXA-induced atrophy. Conversely, CR supplementation sinergically enhanced DEXA diabetogenic effects.

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P.127

TNF-α inhibits the activation of the MAPK family in mammalian skeletal muscles via a COX dependent pathway

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Ageing and some chronic illnesses such as AIDS, chronic obstructive pulmonary disease and cancer are characterised by a slow but progressive loss in skeletal muscle mass commonly referred to as sarcopenia and cachexia, respectively. Recent studies suggest that this muscle wasting may arise from the high titres of circulating pro-inflammatory cytokines associated with these conditions (Rieu et al. J physiol 587:483–5492, 2009; Degens H. Scand J Med Sci Sports 20:28–38, 2010). However, little is known about the cellular-signal transduction events underlying the catabolic effects of these cytokines in mammalian skeletal muscle fibres. Therefore, in this study we

investigated the effects of treating small, intact, skeletal muscle fibre bundles isolated from either the extensor digitorum longus (a fast twitch muscle) or soleus (a slow twitch muscle) of adult mice with the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF-α; also known as cachexin). Our results show that treating the muscle fibre bundles with TNF-α leads to a decrease in the phosporylation of ERK1/2, JNK and p38 well as their downstream effectors, especially c-JUN. Moreover, all of these effects were reversed by pre-treating the muscle fibre bundles with aspirin (a general cyclooxygenase (COX) inhibitor), SC-236 (a selective COX 2 inhibitor) and CAY10526 (a prostaglandin E2α specific inhibitor). From these results we suggest that the catabolic actions of TNF-α in mammalian skeletal muscle are mediated through a COX2 dependent inhibition of the MAPK pathway; and they can be easily reversed by simple interventions such as the use of aspirin.

P.131

Do the decreased *postmortem* pH-values influence the pattern of *postmortem* degradation of the skeletal muscle organ—a histological and fine structural pilot study on rats using different pH-values for fixation

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The delimitation of the time since death is one of the central topics in forensic research. While in the early postmortal phase this temporal delimitation can be fixed by a number of typical characteristics, late postmortal periods bear severe intricacies such as way of death, body composition, environmental changes of the location of the corpse all hamper a confident and reliable delimitation of the time of death. Particularly sensitive is the period between the end of *rigor mortis* and start of degradation processes. Up to now many methods for the early and late *postmortem* phase to delimitate the time since or of death have been published but not all of them are necessarily feasible. So far, to our knowledge no histological or fine structural methods for the delimitation of the time since death have been published, specifically in using skeletal muscle as the target tissue. The main purposes of this study now is to use a pilot model, the rat, (i) to characterize postmortal changes of the skeletal muscle of the hindlimbs using qualitative and quantitative light and electron microscopical analyses and (ii) to assess if the *postmortem* decrease of the pH-values has to be considered or can be neglected for further studies on pigs and human corpses. Thus, we should be able to minimize the number of pigs, optimize the sampling procedure, sample weight (analyses of proteins), fixation protocol (pH-value and osmolarity) as well as to determine the optimal range of post-mortem time-frame to be analysed.

P.132

PGC-1α and PGC-1β, as well as microRNAs-1, -206 and -455, are altered in skeletal muscle of patients with ALS and neurogenic disease

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Amyotrophic lateral sclerosis (ALS) is characterized by the loss of both upper and lower motor neurons, severe muscle weakness and atrophy. Recently, perturbations in the mitochondrial network have been shown to increase muscle atrophy. PGC-1α controls the mitochondrial network and is reduced in several models of muscle atrophy. Therefore the aim of this study was to measure and compare the expression levels of PGC-1α and PGC-1β, as well as several of its known transcriptional targets which regulate mitochondrial biogenesis and function, including NRF-1, NRF2a, TFAM, mitofusin-1, mitofusin-2 and COX-4, in patients with ALS, neurogenic disease (ND) and healthy age-matched controls. We also determined the regulation of several microRNAs involved in muscle development including miR-1, -23a, -133a, -181a, -206 and -455. PGC-1α, mitofusin-1 and COX-4 were significantly reduced in ALS. PGC-1β, mitofusin-2 and NRF-1 were significantly reduced in both ALS and ND. miR-1, miR-206 and miR-455 were increased in both ALS and ND patients. PGC-1α or PGC-1β overexpression in myotubes decreased miR-1 and miR-206. We show that skeletal muscle from ALS and ND patients present perturbations in PGC-1α and PGC-1β and their target mRNAs, as well as muscle specific microRNAs. Our in vitro data suggests a causal link between PGC-1α and PGC-1β and miR-1 and miR-206. The biological relevance of this, with respect to muscle atrophy and function in ALS and ND, requires further investigation.

P.133

Liver and muscle IGF-I expression and signaling molecules in soleus muscle in the course of hindlimb unloading

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The systemic IGF-I level is provided mainly by IGF-I expression in liver (at rest) and muscle (during exercise). Under conditions of unloading the serum IGF-I content declines by 50% (Adams et al. 2002; Litvinova et al. 2007). The present study was purposed to analyze the time-course of IGF-I expression in liver and muscle during hindlimb unloading (HU) and alterations of the downstream signaling molecules. After 3 days of HU we observed threefold decrease of the mRNA of IGF-IEA ($P < 0.05$), but after 7 days of HU the expression rate slightly increased and after 14 days reached $66.9 \pm 13.4\%$ of the control level. We observed the progressive decline of the IGF-IEA expression rate in liver during HU (3 days—$68.3 \pm 13.7\%$; 7 days—$49.0 \pm 9.8\%$, $P < 0.05$; and 14 days—$33.7 \pm 6.7\%$, $P < 0.05$). Thus the drop of serum IGF-I concentration after 14 days of HU may be explained by the decline of IGF-I expression in liver. At the same time, the total content and phosphorylated form of p70S6K (WB) did not exhibit any significant changes untill the 14th day of HU, when they decreased by 20%. We also found the threefold increase of MuRF-1 mRNA amount in soleus after 3 days of HU. The level of MuRF-1 mRNA increased to some extent after 7 days of HU and decreased again by the 14th day (2.2-fold as compared to control). Thus the time-course changes of MuRF-1 expression rate didn't correspond to IGF-IEa expression rate dynamics during HU. The study was supported by RFBR grants 08-04-01557 and 07-04-00763.

P.134

Atrogin-1 regulation in human and mouse skeletal myotubes

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Atrogin-1, an E3 ubiquitin ligase, is increased in numerous models of muscle atrophy and is seen as a potential therapeutic target to combat muscle wasting. While previous rodent studies have consistently shown that under catabolic conditions, Atrogin-1 is regulated by FoXO transcription factors, studies in atrophic human skeletal muscle do not support a dominant role of FoXO. Our aim was to identify potential transcriptional regulators of Atrogin-1 in human and mouse myotubes. Mouse C2C12 and human primary and myotubes were infected with a c-MyC, C/EBPα or PPARδ adenovirus for 48 h. There was no change in Atrogin-1 mRNA following c-MyC over-expression in mouse myotubes. However, Atrogin-1 mRNA levels were increased by 72% and decreased by 52% with PPARδ and C/EBPα over-expression, respectively, mRNA analysis in human myotubes is in progress. There was a 74 and 46% increase in the Atrogin-1 protein following C/EBPα over-expression in both mouse and human myotubes, respectively. Following c-MyC and PPARδ over-expression, Atrogin-1 protein levels increased by 46 and 62% in mouse myotubes respectively, while in human myotubes Atrogin-1 decreased by 23 and 26% respectively. These preliminary results suggest that Atrogin-1 may be transcriptionally regulated by factors other than FoXO, and further highlight that Atrogin-1 regulation is species dependent.

P.135

Genetic ablation of Calsequestrin1 (CASQ1) causes atrophy via activation of proteolytic systems and up-regulation of PGC1 α

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Ablation of Calsequestrin1 in mice causes alteration of calcium transients characteristics and structural alterations of the EC coupling machinery. However, other modifications including atrophy (EDL muscles are 20% smaller than in WT) and increased number/volume of mitochondria have been also detected in adult fast muscles (Paolini et al. 2007). We have performed microarray analysis in fast twitch muscle of WT and CASQ1-null 4-month old mice detecting a significant up-regulation of some "atrogenes", such as Atrogin1, Cathepsin L, and a subunit of the 26S Proteasome. To better define the mechanisms leading to muscle atrophy we studied ER stress response and three proteolytic systems (ubiquitin–proteasome, autophagy and caspase cascade). Increase of calreticulin and HSP70 content suggests the presence of an ER stress condition. Moreover, significant increase of ubiquitinated proteins, and MuRF1 were detected. Whereas, surprisingly the expression of Atrogin1 did not change at the protein level, we found a significant increase of the autophagy marker. In addition, the uncleaved form of caspase3 was also increased. In summary, we have evidences that atrophy in CASQ1-null muscle is likely due to the combined effects of a) activation of the ubiquitin–proteasome pathway and b) increased content of LC3 and caspase3. On the other hand the increased number of mitochondria is likely justified by an increased amount of PGC1α at the protein level which was found in EDL CASQ1-null.

P.137

Role of β^2-adrenoceptors on skeletal muscle atrophy of myocardial infarction-induced heart failure mice

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Sympathetic hyperactivity is a hallmark of heart failure (HF) and it exerts a toxic effect on cardiac muscle. However, less is known about its effects on skeletal muscle in HF. As β^2-adrenoceptors mediate the effect of sympathetic activity in skeletal muscle, we evaluated skeletal muscle structure and phenotype of mice with disruption of 2-adrenoceptor gene (β^2KO) after myocardial infarction (MI)-induced HF. SHAM KO mice and FVB mice (SHAM and MI) were also studied. Fiber typing, cross-sectional area (CSA) and capillary density were evaluated in plantaris muscle. In addition, plantaris chymotripsin-like proteasomal activity was evaluated, since it can be involved in skeletal muscle atrophy in HF mice. β^2KO SHAM mice displayed decreased plantaris mass, reduced CSA of fiber types I, IIA, IIX and IIB, a 44% increased fiber type I and increased capillary density vs. SHAM mice. MI in β^2KO mice caused an additional plantaris atrophy, capillary rarefaction and decreased type I distribution, which culminated in exercise intolerance vs. β^2KO SHAM mice. Proteasome activity was increased in MI mice vs. SHAM. In contrast, β^2KO mice with MI-induced HF displayed decreased proteasome activity in β^2KO mice, which suggest that β^2-adrenoceptors regulate proteasomal activity in skeletal muscle. Taken together, these results suggest that β^2-adrenoceptors are important in counteracting skeletal muscle atrophy in HF at the stage of HF presently studied.

P.138

Knock-down of striated muscle activator of rho signalling (STARS) results in reduced protein synthesis and increased protein degradation

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STARS is a muscle specific actin-binding protein. We have recently shown that STARS is up-regulated in hypertrophied human skeletal muscle following resistance exercise and is decreased following atrophy-stimulating detraining. STARS mRNA is also reduced in sarcopenic mice. These studies suggest that STARS may be involved in skeletal muscle protein synthesis and/or degradation; however this has not been determined. Therefore, to establish its role in protein synthesis and degradation, we used adenoviral infection to overexpress STARS, and siRNA to knockdown STARS, in C2C12 myotubes. STARS over-expression did not influence basal protein synthesis or degradation, nor did it influence insulin stimulated or dexamethasone attenuated protein synthesis. However, knockdown of STARS significantly reduced basal and insulin stimulated protein synthesis by 25%. Additionally, knockdown of STARS significantly increased basal and dexamethasone protein degradation by 20 and 50%, respectively. These observations show that STARS is necessary to maintain the fine balance between basal protein synthesis and

degradation. Furthermore a reduction in STARS may reduce the influence of anabolic stimuli and enhance the protein degradation induced by catabolic stimuli. A minimum amount of STARS may be required to sustain a healthy level of protein turnover.

P.139

Role of sphingolipids pathway in muscle atrophy

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The proinflammatory cytokine TNFα seems to be crucial for muscle wasting in a number of pathological situations, including cancer-induced cachexia. TNFα can induce the synthesis of ceramides, a central molecule of the shingolipid pathway. We set out to investigate the role of sphingolipids in TNFα atrophic effect, by using inhibitors of different steps of the pathway, in vivo and in vitro models of atrophy.

In vitro, TNFα induced an atrophy of C2C12 or L6 myotubes, that was mimicked by exogenous ceramides, and prevented by drugs inhibiting ceramides synthesis. In vivo, we used a well-established model of tumor-induced muscle atrophy, BalbC mice carrying C26 adenocarcinoma, to evaluate the role of sphingolipids. Myriocin, an inhibitor of the de novo pathway of ceramides synthesis, tended to protect animals against tumor-induced loss of body weight and of muscle weight. Moreover, Myriocin treatment significantly reverted the decrease in myofiber size associated with tumor development, and was thus able to protect muscle against atrophy.

These results strongly suggest that ceramides, or a downstream sphingolipid metabolite, are involved in tumor-induced muscle atrophy. Thus, the sphingolipid pathway appears as a new potential target of pharmacological interventions aiming at protecting muscle tissue against atrophy.

P.140

Microgenomics of type 1 and type 2B skeletal muscle fibres

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Skeletal muscle is a complex, versatile tissue composed by a variety of functionally diverse fibre types that differentially express thousand genes. We developed a protocol that allows genomic analysis at single-cell level in mouse muscles (microgenomics) to investigate the expression profiles of slow oxidative (type 1) and fast glycolytic (type 2B) fibres. The comparison between profiles of isolated myofibres and whole muscle clearly shows the advantages of this approach: several non-muscle specific transcripts are subtracted, leading to the detection of a larger collection of muscle fibre-specific mRNAs. Cluster analysis confirms the discriminant power of microarrays, since expression profiles allows a clear division between type 1 and type 2B myofibres. Moreover, metabolic and signalling pathways that are differentially activated in specific fibre types have been identified and several novel genes that could be useful for fibre type functional characterization emerged. Interestingly, among fibres of the same type we observe some differentially expressed genes that have to be confirmed in order to obtain a new high-resolution method for fibre type classification. So, microgenomic technologies have successfully been applied at the level of single muscle fibres and this represents a technological advancement in the field of muscle physiology. This would be of great interest to study muscle plasticity at the fibre level when muscles adapt to new functional demands.

P.141

Myocardial proteome and signaling alterations caused by deficiency in muscle lim protein

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Muscle LIM protein (MLP) could be part of a cardiomyocyte stress-sensor complex, which is impaired in chronic heart disease. In failing human hearts total MLP protein is reduced. Mice with homozygous knockout of MLP ($MLP^{-/-}$) develop hypertrophic or dilated cardiomyopathy 4–8 weeks after birth, but why a lack of MLP promotes cardiomyopathy is unknown. We hypothesized that MLP deficiency alters myocyte hypertrophic signaling. $MLP^{-/-}$ and litter matched $MLP^{+/+}$ mouse hearts were analyzed at 1, 4, and 12 weeks of age, using 2-Dimensional Difference Gel Electrophoresis. About 200 protein spots showed abundance changes, of which 40 proteins were identified by MS/MS. Greatly increased in $MLP^{-/-}$ was four and a half LIM domain protein-1 (FHL1): 14.3-fold at 4 weeks and 12.7-fold at 12 weeks. FHL1 is localized at the Z-disc/I-band, cytosol, and nucleus, where it triggers hypertrophic growth. Other Z-disc associated proteins were elevated in $MLP^{-/-}$: desmin (2.6-fold), cypher (2.7-fold) and calsarcin-1 (2.0-fold). The MLP ligand, cofilin-2 (1.8-fold), and cypher-binding protein, phosphoglucomutase-1 (1.8-fold), were increased in $MLP^{-/-}$, together with their mutual ligand, alpha-cardiac actin (1.7-fold). Metabolic enzymes and small heat shock proteins (HSPs), such as HSPB1 (2.5-fold), HSPB8 (1.7-fold), and alpha B crystallin (CRYAB) (6.6-fold), were elevated in $MLP^{-/-}$. In 7 day old mice, FHL1 (1.4-fold), HSPB1 (2.5-fold), CRYAB (1.6-fold), and desmin (2.6-fold) were already increased. Results were confirmed by immunofluorescence on tissue cryosections and Western blotting of samples separated into subcellular fractions. Further, we found altered isoform expression and distribution of the MLP and calsarcin-1 ligand, calcineurin A.

Preferential alteration of Z-disc proteins in $MLP^{-/-}$ suggests MLP takes part in Z-disc/I-band signaling. MLP deficiency activates the FHL1 and calcineurin hypertrophic signaling pathways.

P.142

Time-resolved analyses of gene expression in a rodent ICU model

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Intensive care unit (ICU) patients commonly develop severe skeletal muscle wasting that aggravate the recovery from the primary disease and weaning from respirator. The modern treatment in anesthesiology and intensive care can progress in Acute Quadriplegic Myopathy

(AQM). This study aims at improving our understanding of the mechanisms underlying the muscle wasting and weakness in ICU patients with AQM. Specific interest is focused on duration-dependent effects on intracellular signaling and myofibrillar gene and protein expression. For that reason, a unique experimental rat model mimicking ICU such as mechanical ventilation, muscle unloading, neuromuscular blocking agents (NMBA) administration and monitoring at different time points from 6 h to 14 days, was used. Gene expression profile was analyzed in gastrocnemius muscle, showing an increased expression in the muscle-specific ubiquitin ligases, atrogin-1 and MuRF-1 after 6 h, as well as other genes involved in translational repression, authophagic/lysosomal genes (LC3b, cathepsins), oxidative stress response and up-regulation of pro-apoptosis signaling, except caspase-3 and caspase-4 that increase after 9 days of intervention. Metalloproteins (methallothioneins, matrix metalloproteinase 11, ADAM family), GST, cystatins, Runx-1, Gadd45a, p21 and Bcl2 are up-regulated in the early stages in response to oxidative stress and cellular damage among other genes, while LIM and sarcomeric proteins, collagen, extracellular matrix transcripts and the muscle-specific calpain-3 were down-regulated mainly after 5 days. These results suggest a very complex, unique and highly temporally coordinated activation of protein synthesis, degradation, protective mechanisms and intracellular signaling during ICU conditions.

P.143

Proteome analysis of skeletal muscle adaptations to 8 and 35-days bed rest

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Human bed rest (BR) is a ground-based model used to simulate the effects of spaceflight on the musculoskeletal system and it is used for the atrophy disuse-induced study. Several open questions still remain about disuse muscle atrophy like the impact of disuse on the metabolic profile and the role of oxidative stress in human muscle. Moreover mechanism underlying to disuse-induced atrophy remain to clarify. In order to get a complete and general view of the changes in expression proteins and their involvement in muscle disuse, we performed a proteomic analysis on vastus lateralis muscle of ten subjects before BR, following 7 and 35 days BR. We found atrophy and decrease of myosin content just at 35 days and we shown a shift of MHCs in the direction MHC-1 → MHC-2A → MHC-2X. The proteomic analysis showed protein changes in antioxidant defence systems, energy metabolism, structural and transport proteins. Among the most relevant results there is the down-regulation of the antioxidant defence systems found at both 7 and 35 days suggesting that oxidative stress could occur in disused human muscle. A changed expression of oxidative and glycolytic enzymes was also found following BR suggesting a general downsizing of energy metabolism; and even the decrease of myoglobin, that plays critical role in energy metabolism, could contribute to the impaired oxidative metabolism. This study supports the idea of an involvement of oxidative stress and enzyme metabolism in disuse atrophy in humans.

P.144

Altered gene expression profile in genetically-induced muscle hypertrophy

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Met-Activating Genetically Improved Chimeric Factor-1 (Magic-F1) is an HGF-derived, engineered protein that contains two Met-binding domains. Magic-F1 protects myogenic precursors against apoptosis, increasing their fusion ability and enhancing skeletal muscle differentiation in transgenic mice (Casano et al.). To deeply investigate gene expression profiles of wt and Magic-F1 satellite cells, microarray analysis has been performed. We described here the preliminary results of microarray analysis focusing on muscular hypertrophy and vasculogenesis gene signatures in satellite cells isolated from Magic-F1 homozygous mice. In parallel we performed functional analysis on transgenic mice that displayed constitutive muscular hypertrophy and accelerating muscle regeneration following injury. Preliminary microarray data show differences in expression of gene related to skeletal muscle homeostasis (growth factors, transcription factors and atrogenes). In particular we observed twofold increase of VEGF-B, MyoD and MURF transcripts in Magic-F1 satellite cells compared to wt. VEGF-B is considered a key regulator for vasculogenesis in skeletal and cardiac muscles (Lohela et al.) Consistently, transgenic mice showed 36.9% increase of muscle capillary network (5 capillaries/fiber on average), in comparison to the controls (4 capillaries/fiber). These findings clearly show that Magic- F1 can positively regulate vasculogenesis, triggering VEGF signaling and increasing the capillary vessel number.

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P.145

Assessing the effects of exercise-induced stress on the Fiona mouse model

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Background and Aim: Characterized by the severe progressive wastage of skeletal muscle, Duchenne muscular dystrophy is a crippling disease that is caused by the absence of the cytoskeletal protein dystrophin. *Utrophin* is a paralogue of *dystrophin*. The Fiona mouse is an *mdx* (dystrophin-deficient) transgenic mouse that over-expresses the full-length utrophin protein in skeletal muscle. Various studies have shown that it is completely rescued and does not display any of the dystrophic characteristics of *mdx* mice. However, these studies have only been performed on sedentary mice. Our aim was to see if Fiona mice continue to display this rescued phenotype after an extended period of sustained exercise-induced stress, or whether they revert to the dystrophic phenotype.
Methods: 4-week-old C57BL/6, *mdx*, and Fiona mice were divided into two groups—'sedentary' and 'run'. Those in the 'run' group were made to run on a treadmill at 12 m min^{-1} for 30 min, twice a week, for 8 weeks. After the end of the trial, muscle samples were dissected out and subjected to a range of tests.
Results: Muscle physiology tests show a significant decrease in maximum isometric force produced by the extensor digitorum longus (EDL) muscle caused by exercise in *mdx* and Fiona but not C57BL/6 mice. Leftward shifts in the force-frequency curves were seen for all groups. Increased centronucleation was seen in muscle sections of

mdx mice but not of C57BL/6 and Fiona. These data indicate that utrophin's protective effect is partially diminished after a sustained period of exercise-induced stress.

P.147

Regulation of muscle dystrophies and protein degradation by the kinome of *C. elegans*

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Kinases transduce extracellular signals to endpoints within the cell. We have employed the nematode *C. elegans*, a validated systems and muscle biology model, to screen the genome for kinases that regulate muscle pathology. As 80% of worm kinases are orthologous to human kinases, this should be a platform to rapidly identify kinases regulating human muscle pathology. A strain containing a muscle lacZ reporter established to read out on cytosolic muscle protein degradation and two GFP strains established to identify dystrophies of myofibres, mitochondria and nuclei were employed. Strains were treated chronically, in replicate, using RNAi feeding vectors with positive results re-examined acutely. We examined 93% of the kinome (RNAi clones for 402 genes available): 13% regulate myofibre morphology, 19% mitochondrial morphology and 28% muscle cytosolic protein degradation. 53% of kinases acutely regulated muscle, suggesting a physiologic and not just congenital role. Most of these kinases are unstudied in man but several are already linked to neuromuscular disease. Although there are limitations, RNAi in *C. elegans* appears to be an efficient method to discover kinases that regulate muscle dystrophies and degradation. Identification of signalling mechanisms by which these kinases regulate muscle atrophy should enhance comparison of the regulation in worm and man.

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P.148

Chronic systemic delivery of low dose morpholino oligomers substantially ameliorates the muscle dystrophic phenotype and normalises activity in mdx mice

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Duchenne Muscular Dystrophy (DMD) is a myodegenerative disorder caused by mutations that create premature termination of dystrophin translation. The administration of antisense oligonucleotides (AOs) to skip one or more exons in mutated forms of the DMD gene and so restore the reading frame of the transcript is one of the most promising approaches to treat the DMD. A similar approach in human would result in the conversion of DMD to the milder Becker Muscular Dystrophy. Due to the transient effect of this treatment, regular administration of AOs would be necessary throughout the patients' lifetime. However at present, preclinical studies demonstrating the efficacy and safety of a long term AO administration have not been conducted. Furthermore, it is essential to determine the minimal effective dose and frequency of administration. The phosphorodiamidate morpholino oligomer (PMO) is one of the most promising AO chemistries thanks to the high affinity to the sequence target and the resistance to endonucleases which reduce the number of administrations and allow a long lasting exon skipping. In this study, two different low doses of PMO designed to skip the mutated exon 23 in the mdx dystrophic mouse were administered for up to 50 weeks. In mice treated for 20 weeks the muscles showed histological and functional improvement. Mice treated for 50 weeks showed a substantial dose-related amelioration of the pathology. Moreover, the generalised physical activity was profoundly enhanced compared to untreated mdx mice showing that widespread, albeit partial, dystrophin expression can restore the normal activity and movement behaviour in mdx mice. Our results show that a chronic long-term administration of low doses of unmodified PMO is safe, significantly ameliorates the pathology and improves the activity of dystrophin-deficient mice, thus encouraging the further clinical translation of this approach in humans.

P.149

Loss-of-function and gain-of-function mutations in actin that cause skeletal muscle diseases act on tropomyosin switching

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We have studied actin filaments isolated from muscle biopsies with two particularly interesting contrasting mutations. The D292V mutation patient had congenital fibre type disproportion (CFTD)2. D292V actin was purified and its presence at 40% of total actin was demonstrated by 2D-electrophoresis. Actin filaments moved normally in vitro motility assay (IVMA) but when tropomyosin was added, mutant filament motility was switched off. It was not possible to reactivate filament sliding by adding troponin in the presence of Ca^{2+} or by adding NEM S-1, which switched on wild-type actin-tropomyosin filaments. The patient with the K326N mutation was reported to have 'stiff' muscles. This phenotype is unique and suggests that thin filaments may be hyperactivated due to the mutation. Actin filaments extracted from a patient biopsy moved normally in IVMA and tropomyosin had little effect upon motility. However, in the presence of troponin, the Ca^{2+}-sensitivity was substantially increased. Thus the D292V mutation tends to inactivate thin filaments whilst the K326N mutation tends to activate thin filaments. Both mutations are within the interface between actin and the alpha band of tropomyosin in the 'closed' state. K326N is a positive to neutral charge change and shifts the equilibrium towards the 'open' state, giving a gain of function. D292V is a negative to neutral charge change and it shifts the equilibrium towards the 'blocked' state, since it is not sensitive to NEM S-1.

P.150

Calcineurin/NFAT-induced expression of IP3R-1 is involved in dystrophin-deficient muscle cell death

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A disregulation of calcium homeostasis seems to be a key element in the physiopathology of Duchenne muscular dystrophy. Here, we investigated the possible involvement of IP3 receptors in spontaneous and in depolarization-induced calcium releases observed in dystrophin-deficient cell lines (SolC1(−)) as compared to mini-dystrophin transfected cells (SolD(+)). Both types of calcium release were significantly reduced after exposure to 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of IP3 receptors. Interestingly, the effect was more pronounced in SolC1(−) than in SolD(+) cell lines. In support with this result, we observed that the expression of IP3-R1 was higher in SolC1(−) than in SolD(+). Treatment of the cells with cyclosporin-A (CsA) or cypermethrine (Cyp), two inhibitors of calcineurin, or with VIVIT, a specific inhibitor of NFAT, reduced the expression IP3-R1 in SolC1(−) to the level of expression observed in SolD(+) cells. It is known that cell survival is largely decreased in SolC1(−) compared to SolD(+) cell line. Interestingly, treatment of the cells with 2-APB, CsA, and Cyp reduced the abnormal rate of cell death observed in dystrophin-deficient cell line. In conclusion, this study shows a possible involvement of calcineurin/NFAT pathway in the disregulation of calcium homeostasis observed in dystrophin-deficient cells. Indeed, pharmacological inhibition of this pathway decreases IP3-R1 expression, reduces calcium releases from the reticulum and protects the cells against cell death induced by the lack of dystrophin.

P.151

Do all patients with *NEB*-based nemaline myopathy share common cellular and molecular alterations leading to muscle weakness?

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Nemaline myopathy is one of the most common congenital myopathies with NEB mutations accounting for approximately 50% of all cases. When exon 55 is deleted, a partial nebulin deficiency occurs, reducing thin filament length. During contraction, this disturbance affects the thin-thick filament overlap, decreasing the number of potential strong interactions between myosin and actin (cross-bridges), thereby, altering cell force production, contributing to in vivo muscle weakness in the patients. It is unknown whether such cascade of events is true when other exons are deleted. In the present experiment, we studied this particular question by using a multidisciplinary approach and biopsy specimens from healthy individuals and a patient with novel NEB mutations. Results demonstrated that exon 3 and exon 22 skippings do not affect nebulin content or thin filament length. Nevertheless, we observed that the nebulin mutations together with a secondary motor protein dysfunction dramatically deregulated the (i) myosin attachment to actin, and (ii) probably thin filament activation, thereby, decreasing the overall number of cross-bridges in the strong binding state and resultant cell force production. This explains, at least in part, in vivo muscle weakness in the patient. Hence, different NEB mutations induce distinct molecular and cellular mechanisms and, consequently, may require different therapeutic interventions.

P.152

Muscle abnormalities in Atlantic salmon

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Tissue malformations in farmed salmon represent not only ethical and welfare issues, but will also negatively affect profitability and product quality due to impaired growth, elevated mortality, and reduced filet quality. We have studied skeletal muscle abnormalities in intensively farmed salmon populations. Several of our observations in the salmon skeletal muscle samples would be defined as pathological in mammals, including moth-eaten myofibres, central positioned nuclei, accumulation of sarcoplasmic masses, ringbinden myofibres, swollen myofibres and degenerative myofibres. Statistical analysis of salmon with information on skeletal deformities, showed linkage between platyspondylii (flattened and compressed vertebrae) and dissolved myofibres near the connective tissue ($P < 0.0047$). In addition, soft fillet texture showed linkage to dissolved myofibres near the connective tissue ($P < 0.032$) and within the muscle tissue ($P < 0.028$). Gene expression analysis on muscle samples showing myofibres with accumulation of sarcoplasmic masses revealed down-regulation of muscle and energy metabolism related genes, but no regulation of immune genes or other genes indicating cause of the muscle damage. Still we do not know the complete impact of the observed muscle abnormalities for fish health and filet quality.

P.153

Efficiency of antisense-mediated exon skipping in normal and mutated DMD genes

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Duchenne and Becker muscular dystrophy (DMD, BMD) are caused by mutations in the dystrophin gene. In general, DMD mutations disrupt the reading frame and lead to prematurely aborted dystrophin synthesis; conversely, mutations that leave the reading frame intact lead to the production of low levels of internally truncated protein, which are sufficient to generate the milder BMD phenotype. This observation suggested that a therapeutic approach for DMD could be based on active intervention on primary RNA processing ("exon-skipping"), aimed at restoring a viable reading frame.

The most commonly used exon-skipping approach relies on the use of sequence-specific antisense oligonucleotides (AO), which binds to intron–exon boundaries and/or to splice enhancer elements.

So far, virtually all AO designing procedures have been tested on intact dystrophin genes. However, there are clear indications that the genomic re-arrangements (deletion, duplications, point mutations, etc.) present in patients can affect the efficiency of the desired skipping processes. In this work we hence decided to compare the efficiency of a set of AO in control and in DMD myoblasts, obtained from patients carrying different types of mutations. Our data indicated that not only AO skipping efficiency varied greatly between intact and mutated DMD genes, but also that at times patients' mRNA exhibited splicing patterns that could not be directly correlated to their specific genomic mutation.

P.154

Silencing SelN disrupts the antioxidant response and misregulates muscle differentiation

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Selenoprotein N (SelN; encoded by SEPN1 gene) is the only selenoprotein associated to a human genetic disease, SEPN1-related myopathy (SEPN1-RM), which is typified by severe weakness and wasting of neck and trunk muscles. Previously we have shown that, in human muscle cells devoid of SelN, there is an increase in intracellular oxidant activity and protein carbonylation, suggesting that SelN plays a key role in antioxidant protection. However, it is still unclear how SelN is involved in antioxidant defence or if SelN has any other cellular roles that could contribute to the patients muscle phenotype. Here we show that the pathogenesis is related to permanent abnormalities in antioxidant defence capacity, and that SelN is involved in muscle differentiation. Using a new in vitro model based on gene silencing, we investigated the main antioxidant defences genes by profiler PCR array. We demonstrated that SelN devoid muscle cells have a specific misregulation of antioxidant genes. We also noted an accelerated rate of differentiation after SelN silencing and demonstrate that SelN silencing modulates MRF and cell cycle exit factors implicating SelN in myogenesis. Our results show that SelN is involved in the regulation of the antioxidant response and suggests that SelN has a novel role in regulating differentiation.

P.155

Myospryn interacts with M-band titin and calpain 3

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The titinopathies tibial muscular dystrophy (TMD) and limb-girdle muscular dystrophy 2J (LGMD2J) are caused by mutations in C-terminal (M-band) titin. Single dominant mutations lead to TMD, and LGMD2J results from the same mutations present on both alleles. Secondary deficiency of calpain 3 (CAPN3) in LGMD2J suggests that the titinopathies may share their pathogenetic pathways with the calpainopathy LGMD2A.

To elucidate the pathomechanisms of TMD/LGMD2J and LGMD2A, we searched for proteins interacting with M-band titin and CAPN3 in yeast two-hybrid screens, and identified myospryn (CMYA5) as a ligand of both proteins. Both novel interactions were supported by coimmunoprecipitation studies of transfected proteins, and coexpression experiments indicated myospryn as an in vitro proteolytic substrate for CAPN3.

Myospryn is a large TRIM-related protein, suggested to function in lysosomal biogenesis and positioning, and in regulating protein kinase A activity in muscle. It has been reported to localize mainly to the costameres, but our microscopic studies on muscle sections and cell cultures demonstrated the localization of endogenous and transfected myospryn also at the M-bands. In addition, in situ proximity ligation assays (PLA) supported the interaction of myospryn with M-band titin.

The novel interactions suggest that myospryn—previously implicated in Duchenne muscular dystrophy and cardiac disease—may also play a role in the pathogenesis of titinopathies and calpainopathy.

P.156

Muscle mitochondrial metabolism and calcium signaling impairment in patients treated with statins

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The most common and problematic side effect of statins is myopathy. To date, the patho-physiological mechanisms of statin myotoxicity are still not clearly understood. In previous studies, we showed that acute application in vitro of simvastatin caused impairment of mitochondrial function and dysfunction of calcium homeostasis in human and rat healthy muscle samples. We thus evaluated in the present study, mitochondrial function and calcium signaling in muscles of patients treated with statins, who present or not muscle symptoms, by oxygraphy and recording of calcium sparks, respectively. Patients treated with statins showed impairment of mitochondrial respiration that concerned mainly the complex I of the respiratory chain and altered frequency and amplitude of calcium sparks. The muscle problems observed in statin-treated patients appear thus to be related to impairment of mitochondrial function and muscle calcium homeostasis, confirming the results we previously reported in vitro.

P.157

Are perisynaptic Schwann cells of neuromuscular junctions affected by spinal muscular atrophy?

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Despite the knowledge about the genetic background of Spinal Muscular Atrophy (SMA), less is known concerning the pathogenetic mechanism leading to the degeneration of neuromuscular junctions (NJ). Among the uncertainties are the patchy informations concerning the ultra-structural changes to NJ provoked by SMA. In the present electron microscopy study, we show modifications of NJ in diaphragm and soleus muscle of 4 day-old SMA-mice ($Smn^{-/-}$; $hSMN2^{+/+}$, pups dying around the 6th day postpartum) compared with muscles of 4 day healthy mice. Interestingly at this developmental stage, the genetic disorder only affects the structure of NJ in

the diaphragm while the soleus is spared. The degenerative NJ show partly retracted axon terminals with focal synaptic contacts, some of which are additionally characterized by swollen mitochondria at both synaptic sides. Surprisingly the perisynaptic Schwann cells of NJ manifest degenerative alterations in form of a condensed cytoplasm and vacuoles in their cellular bodies irrespective of the condition of the mitochondria of the NJ.

Concerning the normal aspect of the soleus, these degenerative structural changes point to possible developmental differences or to destructive effects of the mechanical demand of the highly active diaphragm compared to the soleus, which matures later on. The fact that the perisynaptic Schwann cells are affected, hints to a possible new pathogenetic mechanism, which disturbs the supportive function of these cells for the axon terminal.