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METABOLIC AND SIGNALING EVENTS MEDIATED BY CARDIOTONIC STEROID OUABAIN IN RAT SKELETAL MUSCLE

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Abstract – The cardiac glycoside ouabain initiates a cascade of signaling events through Na^+,K^+-ATPase, leading to an increase in cell growth and proliferation in different cell types. We explored the effects of ouabain on glucose metabolism in skeletal muscle and clarified the mechanisms of ouabain signal transduction. In rat soleus muscle 200~~micro~~M ouabain decreased basal glucose uptake without effect on insulin-stimulated glucose uptake. Ouabain increased glycogen synthesis additively to insulin and this effect was abolished in the presence of a MEK1/2 inhibitor (PD98059) or a c-Src inhibitor (PP2). Ouabain exposure reduced glucose oxidation, and this effect was reversed in the presence of PP2. Incubation with ouabain did not affect intramuscular ATP and its metabolites; however acetyl-CoA carboxylase phosphorylation was reduced, with no effect on AMPK phosphorylation. Insulin-stimulated Akt phosphorylation was not affected by ouabain. Ouabain reduced basal and insulin-stimulated phosphorylation of PKC alpha/beta and delta isoforms, whereas phosphorylation of PKCzeta was unchanged. Ouabain exposure increased interaction of alpha1- and alpha2-subunits of Na-pump with c-Src, as assessed by co-immunoprecipitation with c-Src. Phosphorylation of ERK1/2, GSK 3alpha/beta and p90rsk activity was increased in response to ouabain, and these effects were prevented in the presence of PD98059 and PP2. In conclusion, the cardiac glycoside ouabain stimulates glycogen synthesis additively to insulin in rat skeletal muscle. This effect is mediated by activation of c-Src-, ERK1/2- p90rsk- and GSK3-dependent signaling pathway.

Key words: Ouabain, Na^+, K^+-ATPase, glycogen synthesis, glucose transport, skeletal muscle, extracellular signal-regulated kinase.

INTRODUCTION

Ouabain is one of the cardiac glycosides (obtained from the seeds of *Strophanthus gratus*), which specifically binds to and inhibits the activity of Na^+,K^+-ATPase, a plasma membrane cation pump, which is essential for maintenance of intracellular and extracellular sodium and potassium concentrations, cell volume, osmotic balance and electrochemical gradients (14,26). Ouabain-like compounds cardiotonic steroids (CTS) have recently been described as members of the new class of steroid hormones, endogenously produced in mammalian adrenal glands (36, 37). CTS have been isolated and identified as a constituent of human blood, bovine adrenal glands and hypothalamus (21,37) and circulates in elevated concentrations in blood of 50% of Caucasians with high blood pressure (17). Bovine adrenal cortical cells in tissue culture release CTS in response to norepinephrine, corticotropin and angiotensin II (7, 27, 25). Importantly, exercise causes an acute rise in CTS concentration in the circulation (7,38). Specific binding globulin (μchain of IgM) interacts with CTS and cholesterol (38). Digitalis drugs appear to promote cardiac hypertrophy (12,33). Similarly to other hypertrophic stimuli, ouabain regulates transcription of several hypertrophic marker genes in cardiac myocytes (18,32). Recent evidence shows that in addition to a role in ion transport function, Na^+,K^+-ATPase can sense low concentration of ouabain and play an important role as signal transducer (47). In cardiac myocytes, interaction of ouabain with the Na-pump causes activation of a c-Src, Ras/Raf, p42/44 MAPK signaling pathway, increases

Abbreviations: **ACC**: acetyl-CoA carboxylase; **AMPK**: AMP-activated protein kinase; **GSK**: glycogen synthase kinase; **ERK**: extracellular-signal-regulated kinase; **MAPK**: mitogen-activated protein kinase; **p90 rsk**: p90 ribosomal S6 kinase; **KHB**: Krebs-Henseleit bicarbonate buffer; **TBS-T**: Tris buffer saline with Tween 20.

$[Ca2^+]i$, generates reactive oxygen species in mitochondria, and activates PKCs (47). In the human breast cancer cell line (MDA-MB-435s), ouabain binding to Na-pump induces activation of c-Src, leading to ERK1/2 activation, activation of c-Jun NH2-terminal kinase (JNK), increased level of cell cycle inhibitor p21*Cip1* and growth arrest (22). In renal cells Na^+,K^+-ATPase forms a cell signaling microdomain with inositol 1,4,5-trisphosphate (InsP3) receptor (InsP3R), which in the presence of ouabain, generates slow $Ca2^+$ oscillations (30). Moreover, ouabain stimulates insulin-induced glycogen synthesis, and decreases the production of CO_2 in rat skeletal muscle (10). In this seminal study, cut strips of rat diaphragm, a slow-twitch oxidative muscle were used. However, later research revealed that the strips of diaphragm are leaky for ions, and have increased Na^+,K^+-ATPase activity, depolarized plasma membranes and abnormal ion fluxes. Thus the mechanisms of ouabain signaling in skeletal muscle are unknown.

Because skeletal muscle contains one of the largest pools of Na^+,K^+-ATPase in the body (9), the signal transduction effects mediated by ouabain in skeletal muscle may have a profound metabolic impact. Therefore, the aim of this study was to repeat Clausen's experiment using intact muscle of the same fiber type (slow-twitch oxidative rat soleus) to investigate the effects of ouabain on glucose metabolism in skeletal muscle and to identify the mechanisms of ouabain signal transduction.

MATERIALS AND METHODS

Antibodies and reagents

Ouabain was obtained from Sigma (St Louis, MO, USA). Insulin (Actrapid) was from Novo Nordisk (Denmark). MEK1/2 inhibitor PD98059 and c-Src inhibitor PP2 (4-amino-5-[4-chlorophenyl]-7-[tbutyl] pyrazolo[3,4-d]-pyrimidine) were both from Calbiochem (San Diego, CA). Rabbit polyclonal antibodies to phospho-GSK3 α/β (Ser21/9), phospho-AMPK (Thr172), phospho-PKC α/βII (Thr638/641), δ (Thr505) and ζ/λ (Thr410/403), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAP kinase (Thr180/Tyr182), phospho-c-Src (Tyr416) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to phospho-ACC (Ser79) were from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal antibodies to phospho-ERK1/2 (Tyr204) and phospho-Akt/PKB (Ser473) were from New England Biolabs Inc. (Beverly, MA). Rabbit polyclonal antibody to c-Src was from Santa Cruz Biotechnology, Inc. Mouse monoclonal and rabbit polyclonal antibodies to α1-subunit of Na^+,K^+-ATPase were a kind gift from Dr. M. Caplan (Yale University, New Haven, CT). Mouse monoclonal antibody to α2-subunit of Na^+,K^+-ATPase was a kind gift from Dr. K. Sweadner, rabbit polyclonal antibody to α2-subunit of Na^+,K^+-ATPase was the kind gift from Dr. T. Pressley. Rabbit polyclonal antibody to p90 rsk was a kind gift from Dr. D. Alessi. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G was obtained from Bio-Rad Laboratories (Hercules, CA). Protein A Sepharose CL-4B was from Amersham Biosciences AB (Uppsala, Sweden) and protein G-sepharose was from Sigma (St. Louis, MO, USA). Protein A, horseradish peroxidase linked was from Amersham Biosciences UK Ltd (Buckinghamshire, England). Reagents for enhanced chemiluminescence were obtained from Amersham (Arlington Heights, IL). Solvent NEF-910 G was from Du Pont de Memours GmbH (Hamburg, Germany). All other reagents were of analytical grade (Sigma). *Animals:* Male Wistar rats (110–120g) were purchased from B & K Universal (Sollentuna, Sweden) and housed at the animal facility at the Karolinska Institute. Rats were maintained on a 12hrs light-dark cycle and given free access to standard rodent chow and water. Rats were studied after a 4-5hrs fast. The regional animal ethical committee approved all experimental procedures.

Glucose transport

All incubation media were prepared from a pre-gassed (95% O2/5% CO2) stock of Krebs-Henseleit bicarbonate buffer (KHB), supplemented with 5mM HEPES and 0.1% RIA grade bovine serum albumin (fraction V, RIA grade, Sigma). Pre-incubation media contained 5mM glucose and 15mM mannitol. Rat soleus muscles were pre-incubated in the presence or absence of insulin (0.6nM or 12nM) and ouabain (200μM) for 40min, then rinsed in the media, containing 20mM mannitol for 10min and kept in the hot media with 8mM 3-O-methylglucose with 438μCi/mmol [3H]-3-O-methylglucose for 12min. Muscles were incubated in 2ml of media in a shaking water bath at 30°C. The gas phase in the incubation vial was maintained at 95% O2 / 5% CO2. After incubation in the hot media muscles were frozen in liquidN2 and then processed as described previously (43). When present, insulin and ouabain were maintained throughout the experiment.

Photolabelling of cell-surface glucose transporters

Total cell-surface glucose transporters labeling was performed using biotinylated ATB-BMPA {4,4'-O-[2-[2-[2-[2-[2-[6-(biotinylamino) hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoroethyl) benzoyl]amino-1,3- propanediyl bis-D-mannose}. The utility of this compound has been described previously (23). Following 40min of pre-incubation as described for glucose transport, soleus muscles were transferred to a dark room and incubated for 8min at 180°C in the media containing 1mCi/ml ATB-[2-3H] BMPA. Muscles were irradiated with UV light for 2x3min. Thereafter, muscles were blotted, trimmed free of connective tissue and frozen in liquid N2. Muscles were processed and samples were analyzed as described previously (34, 35) for the determination of cell-surface glucose transporters. Thereafter, proteins were separated by SDS/PAGE and photolabelled glucose transporters were visualized using antibodies to GLUT1 or GLUT4.

Glucose oxidation

Incubation media were prepared from a pre-gassed (95% O_2 / 5% CO_2) stock of Krebs-Henseleit bicarbonate buffer (KHB) (24); supplemented with 5mM HEPES and 0.1% RIA grade bovine serum albumin (fraction V, RIA grade, Sigma). Incubation media contained 5mM glucose, 15mM mannitol and 8mM [U-14C]-glucose (0.2μCi/ml). Rats were anesthetized with 60mg/kg body weight of sodium

pentobarbital. Soleus muscles were dissected for *in vitro* absence of insulin (12nM), ouabain (200µM), MEK1/2 inhibitor PD98059 (22µM) or c-Src inhibitor PP2 (20µM) for 1hr in a sealed flask with a rubber stopper containing a hanging center-well. When present, insulin and ouabain and kinase inhibitors were maintained throughout the experiment. Glucose oxidation and glucose incorporation to glycogen were assesses as described (5). After 1hr, muscles were frozen in liquid N_2 for further analysis of [U-^{14}C]-glucose incorporation into glycogen and vials were resealed immediately. 0.2ml of solvent NEF-910 G was injected into the ~~central~~ well and 0.5ml of 15% PCA was injected into the media. Vials were incubated for an additional 1 hour. The central-well was removed and placed into the scintillation vial, containing 4ml of scintillation fluid and 100µl of 5N HCl. Vials were covered with lids, and placed in the cool dark room for 1hr. Samples were counted for [^{14}C] in the β-counter. Glucose oxidation rate was expressed as nmol/g/hr.

Glucose incorporation into glycogen

Muscles frozen in glucose oxidation experiment were weighed and dissolved in 0.5ml of 1N NaOH at 70°C for 30min in thermomixer. 0.5ml of 20% TCA was added and samples were vortexed and centrifuged at 3500g at 10°C for 15min. The supernatant was transferred to the new tube, and 200µl of glycogen solution (100mg of glycogen in 5ml of distilled water) and 2ml of 95% ethanol were added. Each tube was vortexed and placed at -20°C for at least 1hr or until the contents was fully dissolved, then centrifuged at 2000g for 15min. The supernatant was discarded and 0.7ml of distilled water was added into the tube to dissolve the pellet. Aliquots (500µl) were put into scintillation vials and counted in the β-counter. Glucose incorporation into glycogen was expressed as nmol/g/h.

Western Blot analysis

Parts of frozen muscle samples from glucose oxidation, glycogen synthesis experiments were pulverized in liquid N_2 and then lysed in 300µl of homogenization buffer (137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 20mM Tris pH 8.0, 1% Triton X-100, 10% v/v glycerol, 10mM NaF, 0.5mM Na3VO4, 5 µg/ml leupeptin, 0.2mM PMSF, 5µg/ml aprotinin and 1µM microcystin) on the rotation wheel at 40°C during 1hr. Then samples were centrifuged at 12 000g for 10min, 4°C. Supernatants were collected and protein concentration was measured by the Bradford method (Pierce, Rockford, IL, USA). Samples were prepared for SDS-PAGE (7.5% resolving gel). Proteins were transferred to PVDF membranes and blocked with 7.5% non-fat milk in TBS-T. Membranes were incubated with primary antibodies to p-GSK3 α/β, p-ERK1/2, α1-subunit and α2-subunit of Na^+,K^+-ATPase, p-Akt (Ser473), p-ACC, p-AMPK, p-PKC α/β, δ, ζ isoforms overnight at 4°C on a shaking platform. Membranes were washed with TBS-T and incubated with anti-rabbit or anti-mouse secondary antibody conjugate with HRP. Proteins were visualized by ECL and quantified by densitometry.

Adenine nucleotides measurements

Freeze-dried muscle samples were dissected free from connective tissue, blood and fat and then weighed (around 1.5mg) and extracted in 1.5M PCA before being neutralized with $KHCO_3$. Concentrations of ATP, ADP, AMP and IMP in the samples as well as in external standards were analyzed with HPLC using a reverse phase column (Hypersil Elite, C18, 250x4.6mm, 5µm), as described (39). incubation at 300°C in the presence or Separation of nucleotides was achieved with a flow rate of 1.0ml/min, UV-detection at 254nm and an oven temperature of 40°C.

Immunoprecipitation

Soleus muscle lysates (500µl) were incubated with 40µl of protein Asepharose beads, with rotation for 30min at 4°C. After brief centrifugation, supernatants were collected and immunoprecipitated with antibodies to c-Src overnight at 4°C. Immunoprecipitates were collected on protein A-sepharose beads for 2hrs at 4°C. Beads were washed 3 times in homogenization buffer and twice in ice-cold PBS. Pellets were resuspended in Laemmli sample buffer.

p90 rsk activity assay

Soleus muscles were incubated with 200µM ouabain, in the presence or absence of inhibitors PD98059 and PP2. Muscle lysates (250µg protein/ sample) were immunoprecipitated at 4°C overnight with anti-p90 rsk antibody, previously equilibrated with protein G-sepharose in homogenization buffer. Immunoprecipitates were washed 3 times in homogenization buffer, containing 0.5M NaCl and twice in buffer B (50mM Tris-HCl, pH 7.5, 0.03% Brij-35, 0.1mM EGTA, 0.1% β-mercaptoethanol). Samples were resuspended in 30µl of kinase buffer (50mM Tris-HCl, pH 7.5, 0.1mM EGTA, 0.1% β-mercaptoethanol, 17µM cAMPdependent protein kinase inhibitor peptide, 16.7mM $Mg(Ac)_2$, 50µM cross-tide, and 2µCi of [γ- ^{32}Pi]ATP) and incubated at 30°C for 10min. The reactions were terminated on ice by addition of sample buffer (125mM Tris, 6M urea, pH 6.8). Reaction products were resolved on a 40% acrylamide gel, and ^{32}Pi incorporation into peptide substrate was analyzed by exposing gels to phosphoimager (Fuji BAS-1800II).

Statistics

Data are presented as mean ± SE. Comparisons between groups were performed using Student's t-test. Significance was established at $p < 0.05$.

RESULTS

Glucose uptake and glucose transporters cell surface content

Soleus muscles from male Wistar rats were incubated *ex vivo* and [3H]-3-0-methylglucose uptake was measured in the presence or absence of 200µM ouabain under basal and insulin-stimulated conditions. Ouabain exposure for 1hr decreased basal [3H]-3-*O*-methylglucose uptake by 27%. Insulin-stimulated glucose uptake (0.6 and 12nM) was not affected by ouabain (fig. 1A). Preincubation of rat soleus muscles with 2 mM ouabain was without affect on insulin-stimulated glucose uptake (8.32 ± 1.206 vs 8.5 ± 1.169µM/ml/hr), while basal glucose uptake was reduced (2.32 ± 0.464 vs 1.174 ± 0.238µM/ml/hr, $p < 0.05$). In line with glucose transport data, cell surface glucose transporter photolabeling technique with Bio-LC-ATB-BMPA revealed decreased basal cell-surface GLUT1 content,

without changing cell-surface GLUT4 content (fig. 1B).

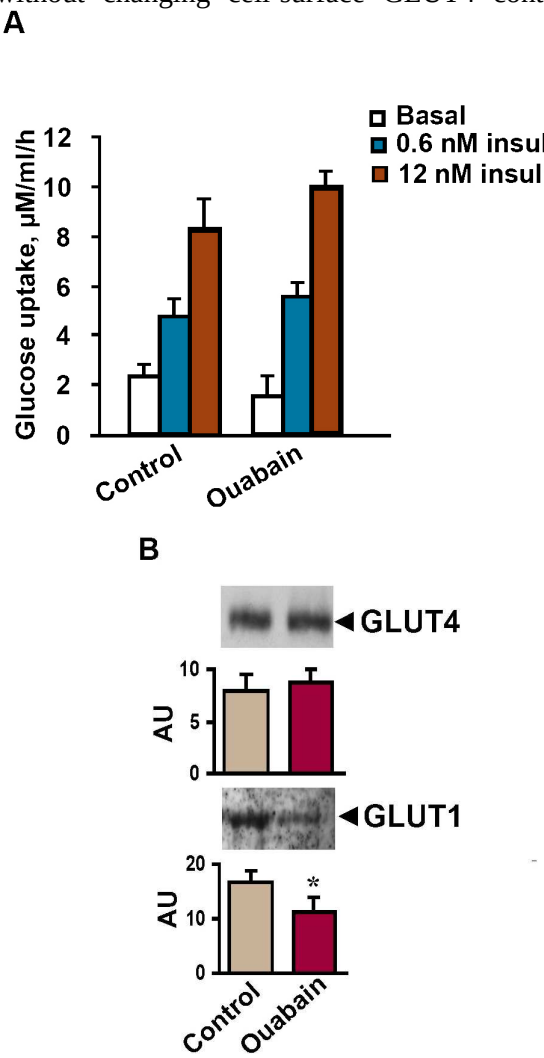

Fig. 1 Effect of ouabain on glucose uptake in rat soleus muscle. A. [3H]-3-O-methylglucose uptake was measured as described in "Materials and methods". Rat soleus muscles were incubated in the presence or absence of 200µM ouabain under basal or insulin-stimulated (0.6nM and 12nM) conditions. Values are mean ± S.E., n = 6. B. Photolabelling of cell-surface glucose transporters. Total cell-surface ATB-BPMA labeling was performed as described in "Materials and methods". GLUT1 and GLUT4 contents was assessed in rat soleus muscles in the presence or absence of 200µM ouabain. n = 4.

Glucose incorporation into glycogen and glucose oxidation

Rat soleus muscles were incubated *ex vivo* to assess glycogen synthesis and glucose oxidation. [U-14C] glucose incorporation into glycogen was stimulated significantly by 200µM ouabain under basal or insulin-stimulated conditions ($p<0.05$). Ouabain stimulated glycogen synthesis additively to insulin (fig. 2A). The effect of ouabain was abolished by inhibitors of MEK 1/2 (PD98059) and c-Src (PP2), under basal or insulin-stimulated conditions (fig. 2A). The ouabain-induced increase in glycogen synthesis was accompanied by a decrease in glucose oxidation under basal and insulin-stimulated conditions (fig. 2B) ($p<0.05$). The decrease in glucose oxidation by ouabain was restored to control levels in the presence of c-Src inhibitor PP2 (fig. 2B).

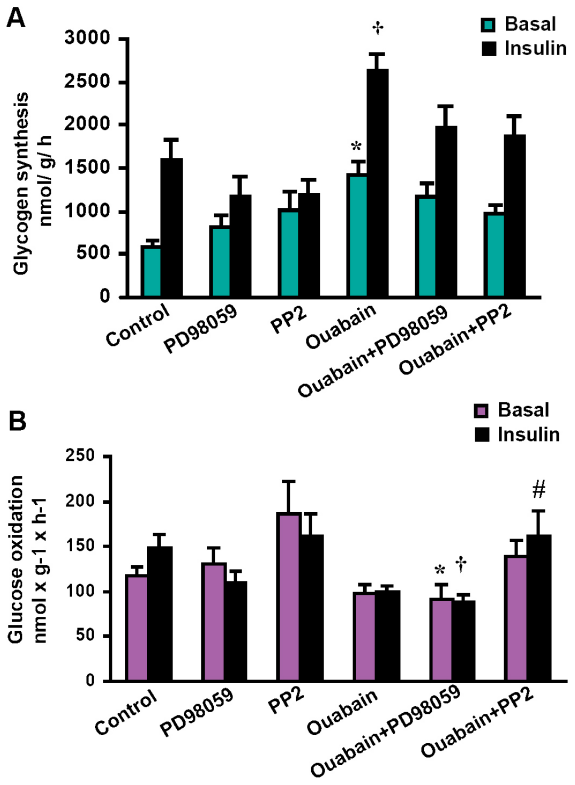

Fig. 2 Effect of ouabain on glucose incorporation into glycogen and glucose oxidation. Rat soleus muscles were incubated in the presence or absence of 200µM ouabain and kinase inhibitors 22µM PD98059 and 20µM PP2 under basal or insulin-stimulated (12nM) conditions. [U-14C] glucose incorporation into glycogen (A) and glucose oxidation (B) were measured as described in "Materials and methods". Values are mean ± S.E., n=11. * $p<0.05$ vs basal, † $p<0.05$ vs insulin-stimulated without ouabain, # $p<0.05$ vs insulin-stimulated in the presence of ouabain without an inhibitor.

Signal transduction pathways activated by ouabain

Since ouabain in concentrations used in our experiments may inhibit Na^+,K^+-ATPase activity, we considered that levels of intracellular ATP and its metabolites may change in the presence of ouabain. However, levels of ATP, ADP, AMP and IMP were unaltered after 2hrs incubation of soleus muscle with 200µM oaubain (fig. 3A). We utilized SDS-PAGE and Western Blotting to evaluate molecular mechanisms of ouabain-dependent signal transduction. Ouabain was

without effect on AMPK phosphorylation, under basal and insulin-stimulated conditions (fig. 3B).

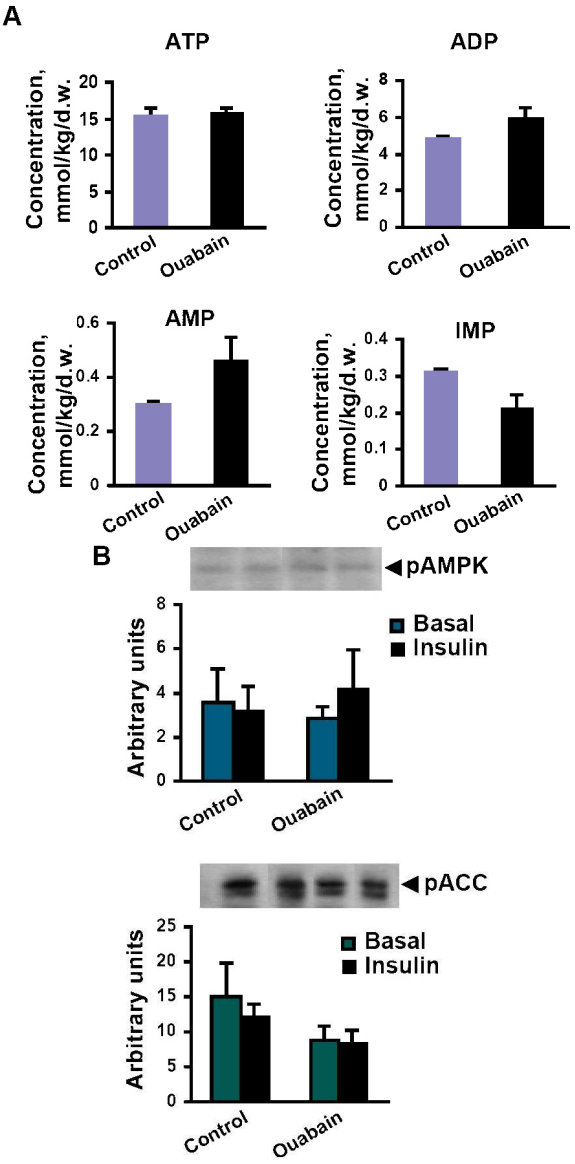

Fig. 3 Effect of ouabain exposure on ATP metabolism in rat soleus muscle *ex vivo*. Muscles were incubated for 2hrs in the presence or absence of 200μM ouabain. A. Muscle ATP and ATP metabolites contents. B. AMPK and ACC phosphorylation. Values are mean ± S.E. n=6. * p<0.05 vs basal.

The Akt/PKB signaling pathway is one of the main pathways regulating glycogen synthesis in skeletal muscle. Glycogen synthase kinase 3, GSK3, a gatekeeper of glycogen synthesis is a down-stream target of Akt/PKB. Insulin-stimulated phosphorylation of Akt/PKB was not affected by ouabain, however basal Akt/PKB phosphorylation was reduced 2.4-fold in the presence of 200μM ouabain (p<0.05) (fig. 4A). Therefore, ouabain signaling is likely to involve other pathways, leading to increased glycogen synthesis. In cardiac myocytes, ouabain increases PKCs phosphorylation and activity (47). Contrary, in rat soleus muscle, ouabain reduced phosphorylation of PKCα/β and PKCδ isoforms (p<0.05) in basal and insulin-stimulated conditions (fig. 4C and D), whereas phosphorylation of PKCζ was unchanged in the presence of ouabain (fig. 4B). To assess whether incubation of muscles with ouabain causes any intramuscular stress, we evaluated phosphorylation of p38 and c-Jun MAP kinases. Phosphorylation of both these kinases was unchanged following ouabain exposure (fig. 4E and F).

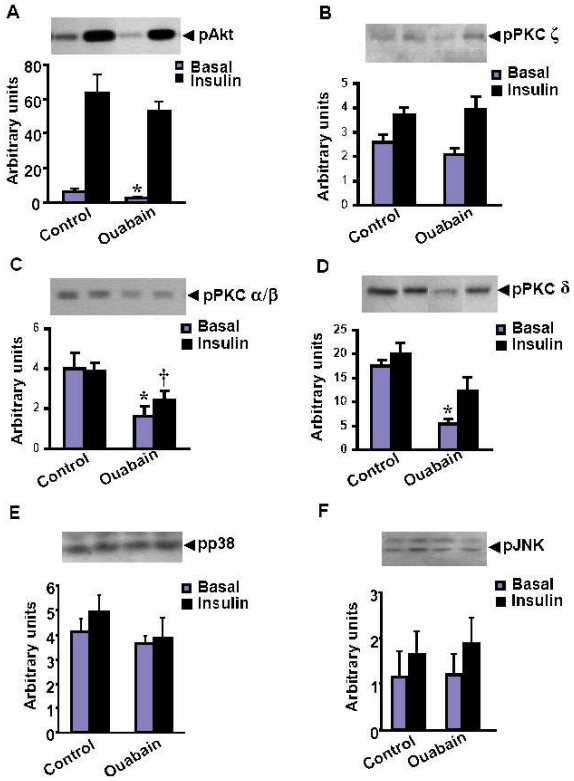

Fig. 4 Effect of ouabain on cell signaling in rat soleus muscle. Muscles were incubated in the presence or absence of 200μM ouabain. A. Akt/PKB phosphorylation. B, C and D. PKC isoforms phosphorylation (ζ, α/β and δ). E and F. p38 and JNK MAP kinases phosphorylation. Values are mean ± S.E. n=6 * p<0.05 vs control basal. † p<0.05 vs insulin-stimulated without ouabain.

c-Src (protein of Src family of tyrosine kinases) is involved in ouabain-induced signalling through Na^+,K^+-ATPase (22,46). We demonstrated that exposure of muscles to 200μM ouabain leads to a profound increase in Src phosphorylation on Tyr416 (fig. 5A). To further investigate c-Src interaction with Na^+,K^+-ATPase, we immunoprecipitated c-Src from soleus muscle lysates and analyzed co-

precipitated proteins by Western blot. Both α1-immunoprecipitated with c-Src under basal and insulin-stimulated conditions (fig. 5B and C).

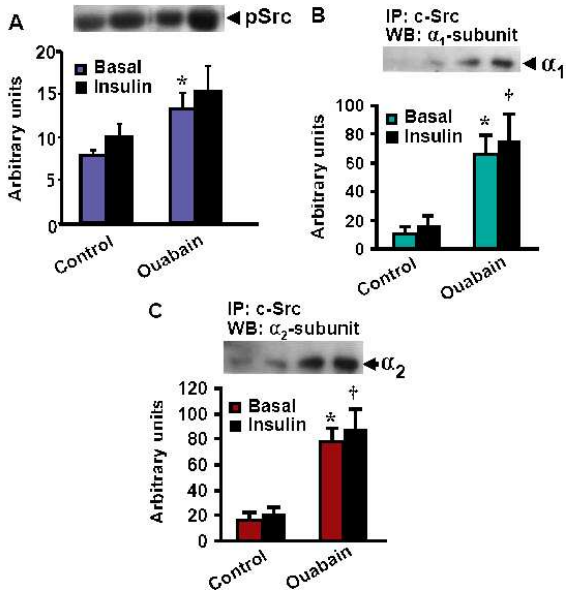

Fig. 5 Involvement of c-Src in ouabain signaling. A. Src phosphorylation in response to 200μM ouabain. B. Na+,K+-ATPase α-subunits co-immunoprecipitation with c-Src. Rat soleus muscles were incubated in the absence or presence of 200μM ouabain, under basal or insulin-stimulated (12nM) conditions. Muscle lysates were immunoprecipitated with anti-c-Src antibodies as described in "Materials and methods". Representative Western blots (upper panels) and quantitative data (mean ± S.E.) (lower panels) for α1-subunit of Na+,K+-ATPase (A) and α2-subunit of Na+,K+-ATPase (B) are shown. n=4 * p<0.05 vs basal, † p<0.05 vs insulin-stimulated without ouabain.

Thus, ouabain is likely to promote direct interaction of Na^+,K^+-ATPase with c-Src molecules, with subsequent phosphorylation of Src, which leads to Src activation. c-Src activation leads to activation of MAPK signaling cascade through Shc, Grb2, Ras, Raf/MEK proteins. Several reports (47,13) provide evidence for MAPK signaling in response to ouabain binding to the Na-pump in various cell types. We found that in soleus muscles ouabain led to a significant increase in basal and insulin-stimulated phosphorylation of MAP kinase ERK1/2 (p<0.05) (fig. 6A).

Phosphorylated ERK 1/2 can activate p90 ribosomal S6 kinase (p90 rsk). Here we provide evidence that ouabain increases p90 rsk activity in rat soleus muscle (fig. 6B). This effect was abolished in the presence of PD98059 and PP2. One of the downstream targets of p90rsk protein kinase is GSK3. In rat soleus muscle phosphorylation of GSK3 was increased by ouabain under basal and insulin-stimulated and α2-subunits of Na-pump were co-conditions (fig. 7A). GSK3 phosphorylation was decreased to basal levels in the presence of inhibitors of MEK 1/2 (PD98059; fig. 7B) and c-Src (PP2; fig. 7C). Thus, we propose that ouabain stimulates glycogen synthesis via c-Src-ERK 1/2 – p90 rsk – GSK3 signaling pathway.

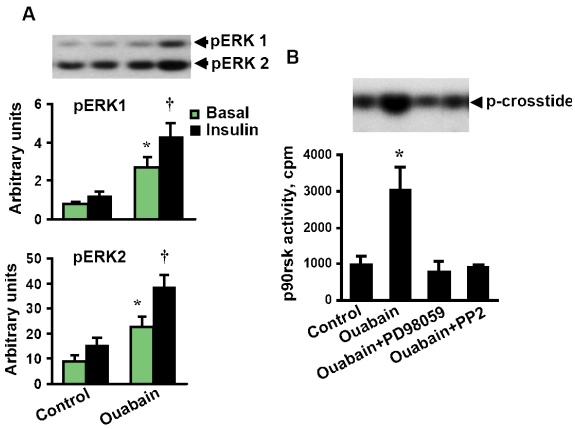

Fig. 6 Effect of ouabain on ERK1/2 phosphorylation (A) and p90rsk activity (B). Rat soleus muscles were incubated in the presence or absence of 200μM ouabain, and inhibitors 22μM PD98059 and 20μM PP2 under basal or insulin-stimulated (12nM) conditions. Muscle lysates were analyzed by Western Blot and p90rsk activity assay as described in "Materials and methods". Representative blots and autoradiogramm (upper panels) and quantitative data (mean ± S.E.) (lower panels) are shown. n=6, * p<0.05 vs basal without ouabain, † p<0.05 vs insulinstimulated without ouabain.

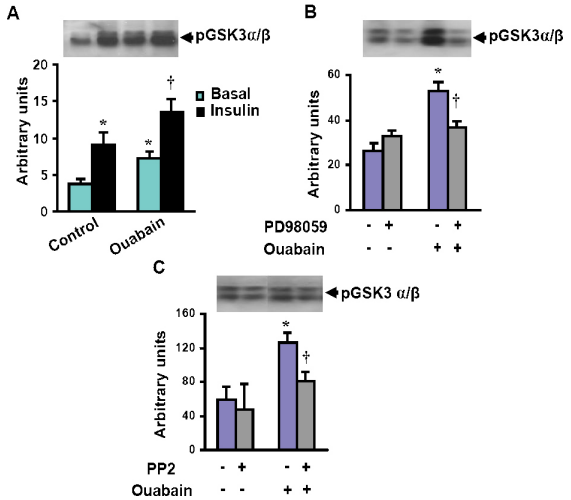

Fig. 7 GSK3 phosphorylation in response to ouabain in rat soleus muscle. A. GSK3 phosphorylation at 200μM ouabain. Representative blot (upper panel) and quantitative data (mean ± S.E.) (lower panel) are shown. n=6, *p<0.05 vs basal, † p<0.05 vs insulin-stimulated without ouabain. B. GSK3 phosphorylation at 200μM ouabain in the presence or absence of PD98059. C. GSK3 phosphorylation at 200μM ouabain in the presence or absence of PP2. n=7 *p < 0.001 vs conrol, † p < 0.01 ouabain + inhibitor vs ouabain without the inhibitor.

DISCUSSION

Ouabain, a digitalis-like cardiac glycoside, is specific inhibitor of Na^+,K^+-ATPase. For almost 200 years glycosides extracted from *Digitalis purpurea* were successfully used to treat patients with heart failure and other cardiac disorders. A role for digitalis-like drugs to improve cardiac contractility and rhythm is well-established, since inhibition of Na-pump leads to an increase in $[Na^+]i$, and an elevation of $[Ca^{2+}]i$ due to the activation of the Na^+-Ca^{2+} exchanger (3,28). In addition to this well characterized positive ionotropic effect, recent reports (1,13,18,22,30,44) provide evidence for a novel mechanism of ouabain-dependent signaling in cardiac myocytes and kidney cells. However, whether ouabain acts as a signal transducer in skeletal muscle, still remains unclear.

We provide evidence that in intact rat soleus muscle *ex vivo* ouabain (200μM) increased [U-14C] glucose incorporation into glycogen under basal and insulin-stimulated (12nM) conditions. Interestingly, under basal conditions glycogen synthesis is increased even when glucose transport into muscle is reduced. The ouabain-induced increase in glycogen synthesis was additive to insulin and diminished in the presence of either the MEK1/2 inhibitor PD98059 or the c-Src inhibitor PP2. Rodent α1-subunit isoform is resistant to ouabain (41). Therefore the concentrations of ouabain used in experiments with rat muscles were much higher than concentrations of CTS observed under physiological conditions. Most likely, in our experimental conditions the majority of α2-subunits will be present in ouabain bound form, whereas only part of α1-subunits will bind ouabain. However, incubation of rat soleus muscle in potassium-free media, where Na^+,K^+-ATPase is inactive, does not stimulate glucose incorporation into glycogen and GSK3 phosphorylation (data not shown). Therefore, our data provides evidence that in rat soleus muscle Na^+,K^+-ATPase plays an important role as a signal transducer and interaction of ouabain, even with a fraction of skeletal muscle Na-pumps, leads to activation of glycogen synthesis.

AMP-activated protein kinase (AMPK), one of the major cell fuel sensors, has been implicated in glucose and glycogen metabolism in skeletal muscle (6). Since the sodium pump is one of the most significant ATP consumers in the cell, we initially suspected that even partial inhibition of Na^+,K^+-ATPase by ouabain may lead to changes in intracellular [ATP] and its metabolites, affecting AMPK activity. However, exposure of rat soleus muscle to ouabain did not change ATP, ADP or AMP levels and AMPK α-sibunit phosphorylation, while phosphorylation of ACC, one of the major substrates of AMPK, was reduced in the presence of ouabain, suggesting decrease in fatty acids oxidation and possible decrease in AMPK activity. Knockout of AMPK catalytic α-subunits leads to decrease in glycogen content in mouse skeletal muscle (20).

However, despite a possible inhibitory effect on AMPK activity and fatty acid oxidation, ouabain stimulates glucose incorporation into glycogen in rat soleus muscle. c-Src has been implicated as a key molecule in ouabain signaling pathway (4,16,22,46,48). In rat soleus muscle ouabain stimulates c-Src phosporylation on Tyr416 residue. The α1- and α2-subunit of Na^+,K^+-ATPase co-immunoprecipitates with c-Src in response to ouabain.

Relatively high α2-subunit abundance in co-immunoprecipitates may reflect a high sensitivity of rodent α2 to ouabain. Our results provide clear evidence that the α2-, and not only the α1-subunit of Na^+,K^+-ATPase - may transmit ouabain signal. Thus, ouabain binding to plasma membrane Na-pump triggers an interaction of the pump with c-Src, and this interaction constitutes one of the first signaling events in response to ouabain (47,48). Interaction of Na^+,K^+-ATPase with Src homology domain containing proteins has been reported previously (8,19,42). Whether ouabain simulates phosphorylation of α-subunits of Na^+,K^+-ATPase by c-Src in rat skeletal muscle, remains to be elucidated.

Activation of c-Src may lead to activation of the MAPK signaling cascade (47). In mammalian cells, c-Src participates in regulating endocytosis of the PDGFβ receptor – GPCR complex, an event which is required in order for PDGFβ receptor – GPCR complex to associate with p42/p44 MAPK in endosomes (45). In addition, c-Src catalyses the phosphorylation and activation of c-Raf, which leads to an activation of p42/p44 MAPK (29). Ouabain also acts as the potent promoter of growth via ERK1/2 activation in rat kidney epithelial cells (13). The MAPK signaling pathway is activated in response to ouabain in cardiac myocytes, thereby affecting gene transcription and translation (47). Notably, in cardiomyocytes ouabain also activates several PKC isoforms (47). We did not observe PKC phosphorylation in rat soleus muscle in response

to ouabain. On the contrary, we observed a in ouabain treated muscle. Whether a decrease in PKC activity in response to ouabain in rat skeletal muscle is a tissue specific phenomenon, remains to be elucidated.

Our data suggest that ouabain stimulates ERK1/2 phosphorylation under basal and insulin-stimulated conditions in rat skeletal muscle. Other members of MAP kinase family, stress regulated kinases p38 and JNK were not activated by exposure to ouabain in rat soleus muscle. ERK1/2 activation may lead to activation of p90rsk protein kinase. p90 ribosomal S6 kinase (RSK) is an important downstream effector of mitogen-activated protein kinase (15). We have shown that in rat soleus muscle ouabain stimulated p90rsk activity and phosphorylation. Activated p90rsk may phosphorylate GSK3 (40). Glycogen synthase kinase (GSK3) was initially described as a key enzyme involved in glycogen metabolism. GSK3 participates in the regulation of different cell functions, including insulin, growth factors and nutrients signaling, control of cell division and apoptosis, and cell fate during embryonic development. A serine residue near the amino terminus of GSK3 (Ser21) is the main target of Akt/PKB. The same residue is phosphorylated through activation of the MAPK cascade (11). Active GSK3 (dephosphorylated) inhibits glycogen synthase. Phosphorylation of GSK3 leads to activation of glycogen synthase and as a result increases glycogen synthesis. We have shown that in rat soleus muscle and human skeletal muscle cells, ouabain causes a dose-dependent increase in GSK3α/β phosphorylation on the Ser21/9 phosphorylation site both by itself and during insulin-stimulated conditions. GSK3 phosphorylation was inhibited in the presence of the MEK1/2 inhibitor PD98059 and c-Src inhibitor PP2. Akt/PKB phosphorylation however was decreased in response to ouabain under basal conditions, and was unchanged in insulin-stimulated conditions. We conclude that in rat soleus muscle ouabain-induced phosphorylation of GSK3 is caused by activation of the MAPK-p90rsk-signaling cascade. Taking into account that glucose oxidation is decreased in response to ouabain, we hypothesize that ouabain promotes storage of glucose in the form of glycogen in skeletal muscle.

Exercise has been demonstrated to cause an acute rise in CTS concentration in circulation decrease in PKCα/β and PKCδ phosphorylation (7, 38). CTS release from adrenal cortical cells is stimulated by angiotensin II and catecholamines (7). Interestingly, ouabain exposure leads to an increase in the mRNA level of interleukin 6 (2), a cytokine produced by skeletal muscle suggested to be involved in response to acute exercise (31). Therefore, our data on the stimulatory effect of ouabain on glycogen synthesis in skeletal muscle may have physiological relevance, as one of the mechanisms of adaptation to exercise *in vivo*. Ouabain may activate glycogen synthesis immediately after exercise, when insulin levels are low. In conclusion, our studies provide evidence that the Na^+,K^+-ATPase inhibitor ouabain leads to an increase in glycogen synthesis, additively to insulin, in skeletal muscle. The increase in glycogen synthesis is mediated by activation of c-Src-, ERK1/2-p90rsk and GSK3-dependent signaling pathway. This phenomenon may constitute a physiological relevant feedback mechanism of adaptation of skeletal muscle to exercise. Elucidation of the signal transducer function of the Na^+,K^+-ATPase in skeletal muscle may have important clinical implications for understanding of mechanisms of development of muscle fatigue, cardiovascular diseases and complications of diabetes mellitus.

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