The IGF-I Signaling Pathway

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Abstract: The insulin-like growth factor (IGF)-I is implicated in the regulation of protein turnover and exerts potent mitogenic and differentiating effects on most cell types. IGF-I biological actions are mediated by the IGF-I receptor, comprised of two extra-cellular α-subunits, containing hormone binding sites, and two membrane-spanning β-subunits, encoding an intracellular tyrosine kinase. Hormone binding activates the receptor kinase, leading to receptor autophosphorylation and tyrosine phosphorylation of multiple substrates, including the IRS and Shc proteins. Through these initial tyrosine phosphorylation reactions, IGF-I signals are transduced to a complex network of intracellular lipid and serine/threonine kinases that are ultimately responsible for cell proliferation, modulation of tissue differentiation, and protection from apoptosis. This review will focus on the IGF-I receptor structure and function, its intracellular signaling pathways, and some important implications of the activation of the IGF-I signal transduction system in specific tissues.

Key Words: IGF-I receptor, insulin receptor, PI 3-kinase, Akt, ERK.

INTRODUCTION

The insulin-like growth factors (IGFs) and their receptors are implicated in the regulation of protein turnover and exert potent mitogenic and differentiating effects on most cell types. IGF-I and IGF-II belong to an integrated growth factor system that includes three hormones (insulin, IGF-I, IGF-II), three receptors, and six binding proteins [1]. The main "endocrine" action of IGF-I is to mediate the growth-promoting effects of pituitary GH. The so-called "somatomedin hypothesis" [2], resulting from the discovery of the "sulfation factor" by Salmon and Daughaday, proposed that the growth-promoting actions of GH were mediated by a second agent released into the bloodstream by a target of GH action. Indeed, GH is now known to induce the synthesis and release of IGF-I by the liver, thus controlling the growth and differentiation of most tissues in the body. Circulating IGF-I is mostly bound to high affinity IGF binding proteins [3], which protect the hormone from proteolysis and modulate its interaction with the IGF-I receptor.

A significant component of IGF action is due to its autocrine and paracrine actions. The original observation of Tollefsen *et al.* [4] that, after removal of serum-containing growth medium, spontaneous differentiation of C2 muscle cells was accompanied by increased expression of the IGFs, suggested that these hormones may be essential autocrine growth factors for muscle cells. Indeed, multiple muscle cell lines have been shown to rely on autocrine IGF production for appropriate differentiation and protection from apoptosis [5, 6]. Unlike other growth factors, IGF-I stimulates both proliferation and differentiation of cells in culture. However, there is a temporal separation between these effects [7]. In myoblasts, the proliferative response to IGF-I lasts 24-36 h, and is followed by myogenic differentiation. In addition, the differentiation-promoting effects of IGF-I can be demonstrated at relatively low IGF concentrations, whereas at higher concentrations (>100 ng/ml IGF-I or 300 ng/ml IGF-II) the differentiation response appears to decrease progressively. The stimulation of proliferation by IGF-I results in an increase in cell number, protein levels, DNA synthesis, uptake of amino acids and glucose, and a suppression of proteolysis. On the other hand, IGF-I stimulates differentiation by promoting gene expression of regulators of terminal differentiation, such as myogenin in skeletal muscle cells.

The IGFs bind with high affinity to two cell surface receptors, the IGF-I (or type I) receptor, which has a high degree of homology to the insulin receptor, and the IGF-II (or type II) receptor, which has been shown to be identical to the cation–independent mannose 6-phosphate receptor. The type I receptor binds IGF-I with the highest affinity but also binds IGF-II and insulin with approximately 10- and 100-fold lower affinity, respectively. The type II receptor binds IGF-II with the highest affinity, IGF-I with much lower affinity, but does not bind insulin. The IGFs are also able to interact with the insulin receptor, but with much lower affinity. Thus, most of the observed IGF biological effects on cell growth, differentiation and survival depend on the activation of the IGF-I receptor signaling pathway, which shares multiple intracellular mediators with the insulin signaling cascade.

THE IGF-I RECEPTOR

The IGF-I receptor is a hetero-tetrameric protein, consisting of two identical extracellular α-subunits containing a cysteine-rich IGF-binding site, and two transmembrane β-subunits, bearing an intrinsic tyrosine kinase activity, which depends on an ATP-binding site (lysine 1003) and a cluster of three tyrosine residues at positions 1131, 1135, and 1136. Binding of IGF-I to its receptor causes the activation of the tyrosine kinase, leading to autophosphorylation of the intrin-

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sic tyrosines and tyrosine phosphorylation of multiple cytoplasmic substrates. Additional tyrosine residues in the C-terminal domains of the β-subunit are also important in signal transduction by the IGF-I receptor [8]. The functional relevance of distinct domains within the IGF-I receptor β-subunit has been investigated through mutational studies. Mutation of the ATP-binding site [9] and/or substitution of phenylalanines for tyrosines in the 3-tyrosine cluster [10-11] is associated with complete inactivation of the receptor kinase and delayed differentiation in muscle cells [12]. Additionally, the distal tyrosine (position 1316) in the carboxyl-terminal domain of the β-subunit appears to be important for tumor formation, but it is not essential for IGF-I-stimulated mitogenesis [13]. Therefore, it is possible that signaling emanating from the IGF-I receptor to either proliferation or differentiation could be determined at the receptor level by activation of specific tyrosine residues on the β-subunit, recruiting distinct signaling molecules further down the signal transduction pathway.

Insulin and IGF-I functions are physiologically distinct but overlapping. In mammals, insulin acts mainly on carbohydrate, lipid and protein metabolism in muscle, adipose tissue and liver, although long-term effects on the transcription of regulatory enzymes are also important. In contrast, IGF-I promotes cell growth, survival and differentiation [14]. The biological relevance of the IGF system was most strikingly demonstrated when the expression of various molecules (ligands, receptors, and binding proteins) was modified in genetically engineered experimental models. As an example, transgenic animals overexpressing IGF-I show an approximately 30% increase in muscle and bone growth, associated with a substantial increase in both weight and DNA content of spleen, pancreas, brain, and kidney [15]. IGF-I receptor-deficient mice are severely growth-retarded (45% of normal) and die within minutes of birth, probably as a result of impaired development of respiratory muscles. In addition, they are born with multiple abnormalities, including muscular hypoplasia, delayed ossification, and thin epidermis [16]. In addition, fibroblasts isolated from IGF-I receptor knockout mice grow very slowly in culture, unless they are transfected with the IGF-I receptor. On the other hand, mice lacking the insulin receptor are born with modest growth retardation (approximately 10%) [17]. Their embryonic development is otherwise unimpaired. After birth, they rapidly develop diabetic ketoacidosis and die within few days [17, 18]. Therefore, insulin receptor appears to be necessary for postnatal, but not for embryonic, metabolism.

The IGF-I receptor is highly homologous to the insulin receptor at both DNA and protein levels, particularly in the tyrosine kinase domain (84%), but differs markedly in other regions (22-26% homology in the transmembrane domain, 45% homology in the carboxyl-terminal domain) [19]. The analysis of structural features of the two related receptors may help to define the molecular basis of distinct signaling properties of IGF-I and insulin. Indeed, the insulin and IGF-I receptors are relatively specific for their respective ligands, although at high concentrations insulin cross-reacts with the IGF-I receptor and *vice versa*. Both receptors are widely expressed in mammalian tissues, albeit at varying levels. In cells expressing both insulin and IGF-I receptors, pro-receptors undergo hetero- as well as homo-dimerization, creating insulin receptor-IGF-I receptor hybrids together with classical receptors [20-22]. Heterodimers appear to assemble with comparable efficiency to homodimers, and when either receptor is in substantial excess, the less abundant receptor is almost entirely present into hybrids. Thus, in skeletal muscle IGF-I receptors are present largely as hybrids, together with an excess of classical insulin receptors, whereas the opposite occurs in fibroblasts [23]. Hybrid receptors bind both insulin and IGF-I, although they have lower affinity for insulin than classical insulin receptors [24]. Binding of either ligand leads to autophosphorylation, and presumably activation, of the tyrosine kinase intrinsic to both β-subunits [25], but outcomes in terms of biological responses remain to be defined.

Additional complexity of the IGF system arises from distinct structural features and signaling potentials of the two receptors intracellular domains. The insulin receptor cytoplasmic domain bears 13 tyrosine residues, whereas the IGF-I receptor shows 15 tyrosines. Seven out of the 13 tyrosines in the insulin receptor are phosphorylated in response to insulin binding [26], as are eight out of the 15 in the IGF-I receptor [27, 28]. These tyrosine residues are clustered in three different domains: the juxta-membrane, catalytic, and carboxyl-terminal regions. The roles of tyrosine residues in the juxta-membrane and catalytic domains appear to be conserved. However, experimental evidences suggest that intrinsic differences in the catalytic domains may explain signaling selectivity (see below).

THE IGF-I SIGNAL TRANSDUCTION PATHWAY

The IGF-I receptor interacts with and signals to adaptor molecules, namely the insulin receptor substrate(IRS)-1/4 and the Shc (Src homology collagen) proteins (p46/p52/p66), which serve as signaling nodes for distinct intracellular pathways. Both IRS-1 and IRS-2 interact directly with the insulin and IGF-I receptors [29-31], IRS-2 being apparently a better substrate for the insulin than for the IGF-I receptor [32, 33], supporting the view that the biological specificity of the two receptors depends, at least in part, from preferential signaling events [34, 35]. Phosphorylated IRSs recruit and activate class 1a phosphoinositide 3-kinase (PI 3-kinase) *via* the two SH2 domains of the adaptor protein p85, leading to synthesis of membrane-associated phosphorylated inositols. These molecules, in turn, recruit and activate phosphoinositide-dependent kinases (PDKs), which then phosphorylate and activate other protein kinases, including Akt/protein kinase B, p70rsk and protein kinase Cζ (PKCζ) [36]. There is substantial evidence that PI 3-kinase activity mediates a wide range of insulin/IGF effects, including stimulation of glucose transport, glycogen synthesis, protein synthesis, mitogenesis, inhibition of apoptosis, and regulation of gene transcription [37].

Another IGF-I-activated signaling pathway, which is coupled mainly to mitogenic and transcriptional responses, involves recruitment to both IRS-1 and Shc of the guanine-nucleotide-exchange factor Sos, *via* the SH2 domain of the adaptor Grb2. This leads to activation of the small G-protein Ras, which in turn activates the protein serine kinase Raf and the extracellular-signal-regulated kinase (ERK) cascade. Shc may actually compete with IRS-1 for a limited cellular pool

of Grb2, and the extent of Shc/Grb2 binding correlates with the amount of insulin-activated ERK and c-fos transcription [38]. Therefore, the Shc/Grb2 pathway represents the predominant mechanism leading to the Ras/ERK signaling pathway. Recent experimental evidence has shown that distinct Shc isoforms exert opposite effects on the ERK signaling cascade. The Shc proteins originate by alternative use of three distinct translation starting points on a longer transcript ($p46^{Shc}$, $p52^{Shc}$, and $p66^{Shc}$) and two translation starting points on a shorter transcript ($p46^{Shc}$, $p52^{Shc}$); the two mRNA transcripts are generated by alternative splicing from a single gene [39-41]. Although all three Shc isoforms can be tyrosine-phosphorylated upon growth factor stimulation, $p46/p52^{Shc}$ are coupled to growth and survival signals, whereas $p66^{Shc}$ also undergoes serine phosphorylation and mediates pro-apoptotic responses to oxidative stress [42]. Specifically, the $p66^{Shc}$ has been shown to regulate intracellular oxidant levels and hydrogen peroxide-mediated forkhead inactivation [43], effects that are probably relevant to the reported ability of $p66^{Shc}$ to control lifespan in mammals [42] and are unique to this Shc isoform. Therefore, while $p46/p52^{Shc}$ trigger the activation of the ERK pathway *via* Grb2/Sos/Ras, $p66^{Shc}$ has been shown to exert an inhibitory effect on ERK, because reduced expression levels of $p66^{Shc}$ were associated with persistent ERK activation [44]. The opposite effects of $p46/p52^{Shc}$ and $p66^{Shc}$ on ERK activation are of pathophysiological significance, since human breast cancer tissues with high $p46/p52^{Shc}$ to $p66^{Shc}$ expression ratios show increased proliferative activity and are associated with poor prognosis [45].

IGF-I receptor activation is also coupled to the stimulation of a family of MAP kinases, besides ERK-1/2, including Jun kinase (JNK)-1 and -2 [46] and p38 MAP kinase [47, 48]. JNKs phosphorylate the amino terminus of c-Jun, increasing its ability to activate transcription [46]. Multiple targets have been identified downstream the MAP kinases, including ribosomal S6 kinase (Rsk 90), MAPKAP, phospholipase A2, and multiple transcription factors [49]. Recent studies indicate that Akt can inhibit the Raf protein kinase by phosphorylation on Ser^{259}, leading to interaction of Raf with 14-3-3 proteins and inhibition of the Ras/ERK signal transduction pathway [50]. This cross-talk pathway may not operate ubiquitously, as Akt does not inhibit Raf in undifferentiated myoblast precursor cells, but it does when these cells are differentiated into skeletal-muscle myotubes [51].

IGF-I has also been shown to induce tyrosine phosphorylation of the cytoplasmic protein Crk [52], which shares some sequence homology with Grb2 and Nck. Crk has been found to associate with mSOS [53] and may be involved in IGF-I receptor-mediated modulation of the Ras-MAP kinase signaling pathway, thus enhancing IGF receptor-dependent mitogenesis [54-56].

IGF-I-induced phosphorylation of IRS-1 results also in the recruitment of the phosphotyrosine phosphatase Syp through its two SH2 domains, leading to increased phosphatase activity [57] and downregulation of hormone-triggered tyrosine phosphorylation events. PTP-1B and PP-2A represent additional phosphatases involved in the fine regulation of IGF-I/insulin-induced signaling [58]. Other proteins have been shown to interact with phosphorylated IRSs upon IGF-I stimulation, including the phosphotyrosine phosphatase Syp and the SH3-containing adaptor Nck, although their specific role is still uncompletely defined [59].

The preferential association of cytoplasmic proteins with the IGF-I receptor as compared to the insulin receptor may represent an additional mechanism for signaling specificity. The intracellular protein Grb10, that contains both SH2 and pleckstrin homology consensus sequences, has been cloned as an insulin- and IGF-I receptor-interacting protein [60, 61]. Grb10 interacts directly with both the insulin and the IGF-I receptor *in vitro*, although it appears to associate preferentially with the insulin receptor in intact cells [62], due to specific molecular determinants [63]. The role of Grb10 remains obscure, with some studies reporting inhibition of receptor function [64-66] or of specific pathways [67], and others suggesting enhancement of mitogenic responses [68, 69]. Interestingly, mutations in the Grb10 sequence have been found in two patients with Russell-Silver syndrome, characterized by prenatal and postnatal growth retardation and dysmorphic features, suggesting a modulating role of Grb10 in human growth processes [70].

Multiple isoforms of the 14-3-3 proteins have been found to bind to activated IGF-I receptors, but not to insulin receptors, *via* Ser^{1272} and/or Ser^{1283}, which are unique to the IGF-I receptor sequence [71, 72]. The significance of this preferential association remains to be determined, although 14-3-3 proteins have been shown to act as scaffolding proteins involved in the regulation of cellular growth and survival [73].

Finally, different G-proteins show a specific pattern of association with the insulin or the IGF-I receptor. $G\alpha q$ has been shown to be a substrate for the insulin receptor, and has been implicated in insulin signaling to glucose transport [74]; in contrast, IGF-I but not insulin receptor appears to be constitutively associated with Gi, and to induce the release of $G\beta\gamma$ subunits following activation [75, 76].

IGF-I SIGNALING IN SKELETAL MUSCLE AND HEART

IGF-I is essential for anabolic, mitogenic, and differentiation responses in skeletal muscle and myocardium, and these actions require the integrity of the IGF-I receptor signal transduction machinery. Mice lacking a functional IGF-I receptor die immediately after birth of respiratory failure and exhibit a severe growth deficiency (45% of normal size), characterized by general organ hypoplasia including the muscle [16]. By contrast, overexpression of IGF-I receptor in L6 myoblasts is associated with ligand-dependent stimulation of proliferation and differentiation: specifically, a normal differentiation rate is evident at low IGF-I concentrations, whereas at high IGF levels a greatly enhanced proliferation and inhibition of differentiation can be demonstrated. When switched to low serum medium, IGF-receptor-overexpressing C2 myoblasts differentiate rapidly and express very high levels of myogenin, a key differentiation marker for muscle cells [77, 78].

Both the Shc/Ras/ERK and the PI 3-kinase/Akt pathways have been shown to mediate metabolic, mitogenic and myogenic effects in established muscle cell lines and primary satellite cell cultures. Activation of the Shc/Ras/ERK signal-

ing cascade results in modulation of expression and/or activity of multiple transcription factors and other intracellular kinases, leading to increased cell proliferation [79]. On the other hand, PI 3-kinase activation increases the initiation of translation by regulating the phosphorylation state of eukaryotic initiation factor 4 binding protein and the p70 S6-kinase, which in turn enhance the translation of mRNAs encoding ribosomal proteins and elongation factors, integral components of the protein synthesis machinery [80]. In addition, inhibition of PI 3-kinase signaling in satellite cell cultures has been shown to prevent the completion of the cell cycle, inducing arrest in the G1 phase. That would be expected to lead to either cell differentiation or apoptosis [81]. Thus, both pathways may act in concert [82, 83], and both may be required for the differentiation of myoblasts [84]. The relative contribution of the ERK vs. the PI 3-kinase pathway in modulating myoblast biological responses has been highlighted in L6 myoblasts treated with dexamethasone [85]. In these experimental conditions, tyrosine phosphorylation and total cellular content of the IGF-I receptor were found to be significantly increased, whereas IRS-1 phosphorylation and content appeared to be reduced. The decrease in IRS-1 phosphorylation apparently was not associated to reduced mitogenesis, suggesting that other signaling intermediates may play a major role in controlling cellular proliferation. Indeed, in dexamethasone-treated cells, the level of IGF-I-stimulated tyrosine phosphorylation of the three Shc isoforms was significantly higher as compared to control myoblasts, paralleling the augmented mitogenesis in response to IGF-I [85].

Recently, the $p66^{Shc}$ isoform has been shown to exert a specific role in regulating ERK signaling and cell biological functions in cultured myoblasts [44]. L6 myoblasts with a targeted deletion of the $p66^{Shc}$ protein isoform showed an increase in ERK-1/2 phosphorylation under basal conditions and blunted ERK-1/2 stimulation by IGF-I. The reduction of the $p66^{Shc}$ protein resulted in marked phenotypic abnormalities, and was associated with impaired DNA synthesis in response to IGF-I, lack of terminal differentiation into myotubes, and complete disruption of cell cytoskeleton. Reduction of the abnormal increase in ERK-1/2 activation with the specific inhibitor PD98059 restored the actin cytoskeleton and the cell morphology. Hence, the $p66^{Shc}$ protein likely exerts an inhibitory effect on the ERK signaling pathway in myoblasts, which is necessary for maintenance of IGF responsiveness and normal cell phenotype [44].

IGF-I appears to play a critical role in preserving normal myocardial functions and preventing age-and/or stress-related cardiac injuries, including alterations secondary to myocardial infarction [86]. In addition, IGF-I appears to protect myocardial cells from degeneration and cell death, as well as to stimulate cell regeneration, in both animal models [87, 88] and cultured rat cardiomyocytes [89]. The anti-apoptotic actions of IGF-I can be observed at physiological concentrations, and rely on the activation of specific IGF-I receptor signaling pathways. IGF-I activates both ERK and PI 3-kinase pathways in cardiac muscle [90-92]. In particular, activation of the PI 3-kinase/Akt cascade promotes survival of cardiac myocytes both *in vitro* and *in vivo*, and protects cells from reperfusion injury *in vivo* [93, 94].

IGF-I SIGNALING IN THE BRAIN

IGF-I is highly expressed within the brain and participates in the regulation of brain development by promoting neuronal survival and synaptogenesis during early postnatal life. Similarly to what has been described in other peripheral tissues, in neurons insulin/IGF-I receptor activation triggers the activation of the PI 3-kinase/Akt pathway. The active, phosphorylated form of Akt (phospho-serine^{473} and -threonine^{308} Akt) is concentrated in granular deposits in IGF-I-expressing neuronal processes in mouse brains, but is barely detectable in these same neurons in IGF-I null brain [95]. In the control brain, GLUT4 appears to co-localize with phospho-Akt in the processes of IGF-I-expressing neurons, suggesting a physical association of these proteins. In the IGF-I null brain, however, GLUT4 is reduced and essentially localized in perikarya. GLUT4 mRNA is also decreased in IGF-I null neurons [95]. Thus, IGF-I-induced Akt phosphorylation appears linked to both production and translocation of neuronal GLUT4 from intracellular pools to nerve process membranes in the normal developing brain. Hexokinase activity is also significantly reduced in IGF-I null brain, suggesting a role for IGF-I in regulation of brain hexokinase activity, which may also contribute to glucose utilization [95]. These data suggest that IGF-I signaling serves an anabolic, insulin-like role in brain metabolism. Another target of insulin/IGF signaling *via* Akt in the brain is glycogen synthase kinase 3β (GSK3β). The colocalization of phospho-GSK3β with abundant glycogen stores specifically in IGF-I-expressing neurons suggests that IGF-I acts in an autocrine manner to promote glucose uptake and storage as glycogen in developing projection neurons [95]. In the same line of evidence, the phosphorylation of tau, a GSK3β substrate involved in neurofilament stabilization, was found to be increased in the IGF-I null brain compared to wild-type mice, providing further evidence that IGF-I may normally regulate neuronal development by inhibiting brain GSK3β activity [96]. Finally, GSK3β has been implicated as a pro-apoptotic factor in neurons [97-100], thus GSK3β hyperactivity in the IGF-I null brain may contribute not only to hypoplastic neuronal development through reduced anabolic processes, but also to increased neuronal loss.

IGF-I SIGNALING IN β-CELLS

Recently, it has become evident that the receptors for insulin and IGF-I and most proteins in their signaling pathways are present and functional in the insulin-producing pancreatic β-cells [101-106]. The distinct role of the IGF-I receptor vs. the insulin receptor in β-cell growth/function has been recently defined using a sophisticated strategy, consisting in selective deletion of these receptors, *in vivo*, by genetic manipulation. Loss of the insulin receptor in the β-cells leads to altered glucokinase expression in the β-cells and a gradual reduction in the β-cells mass. Both these effects lead to the characteristic feature of a selective loss of glucose-stimulated acute phase insulin release that is typically observed in patients with type 2 diabetes [103]. By contrast, mice with β-cell IGF-I receptor knockout are normal at birth and show no significant differences in islet size, β-cell mass or pancreatic insulin content when compared with controls up to 2 months of age. A mild hyperinsulinemia is evident by

2 months of age; however, blood glucose levels are comparable between the controls and the knock-outs [107]. IGF-I has been shown to inhibit insulin secretion both *in vivo* and *in vitro* [108, 109], thus the hyperinsulinemia in the β-cell IGF-I receptor knockout mice is probably due to the loss of a normal tonic inhibitory effect of IGF-I on insulin secretion. Similar to what observed in the β-cell insulin receptor knockout mice, β-cell IGF-I receptor knockout mice show a loss of acute phase insulin secretion after glucose stimulation and glucose intolerance. Insulin and IGF-I receptors are known to have several common downstream signaling proteins [8, 30, 110], and the presence of similar phenotypes in the β-cell IGF-I receptor knockout and β-cell insulin receptor knockout mice may be explained by the presence of insulin/IGF-I receptor hybrids in the β-cells that can activate either of the signaling pathways. In addition, the factors that control islet/β-cell growth early in embryonic life [111] and during the compensation that occurs during insulin resistance [111, 112] are not fully understood. The presence of normal islet growth and development in the β-cell IGF-I receptor knockout and β-cell insulin receptor knockout mice suggests that these receptors may not be critical in the early development of the islets. However, it is possible that these receptors play a role during the islet hyperplastic response to insulin resistance [112] or in the response to pancreatic injury [113].

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