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IGF-1 receptor as an alternative receptor for metabolic signaling in insulin receptor-deficient muscle cells

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Abstract We have derived skeletal muscle cell lines from wild-type (wt) and insulin receptor (IR) knockout mice to unravel the metabolic potential of IGF-1 receptor (IGF-1R). Both wt and $IR^{-/-}$ myoblasts differentiated into myotubes with similar patterns of expression of muscle-specific genes such as MyoD, myogenin and MLC1A indicating that IR is not required for this process. Binding of ^{125}I-IGF-1 on wt and $IR^{-/-}$ myotubes was similar showing that IGF-1R was not upregulated in the absence of IR. Stimulation of $IR^{-/-}$ myotubes with IGF-1 (10^{-10} to 10^{-7} M) increased glucose uptake and incorporation into glycogen, induced IRS-1 phosphorylation and activated PI 3-kinase and MAP kinase, two enzymes of major signaling pathways. These effects were comparable to those obtained with wt myotubes using insulin or IGF-1 or with $IR^{-/-}$ myotubes using insulin at higher concentrations. This study provides a direct evidence that IGF-1R can represent an alternative receptor for metabolic signaling in muscle cells.

Key words: Insulin receptor; Insulin-like growth factor-1 receptor; Signal transduction; Metabolic action; Muscle cell

1. Introduction

The biological effects of insulin and insulin-like growth factors (IGFs) are mediated by the insulin receptor (IR) and the IGF-1 receptor (IGF-1R) which are very similar heterotetrameric $\alpha_2\beta_2$ receptor tyrosine kinases [1]. Studies concerning specificity and redundancy between IR and IGF-1R in vivo are hampered for a variety of reasons. The two receptors, present on the surface of most of the cells, activate common signaling pathways [2,3] and can bind the heterologous ligands. Moreover, some biological effects might be achieved through IR/IGF-1R hybrid receptors [4]. Mutant mice carrying a null mutation in the genes encoding IR [5,6] or IGF-1R [7] and cell systems derived from these mutants [8–10] represent unique tools for dissecting the specific contributions of IR and IGF-1R, respectively, in cell growth, differentiation and metabolism.

Although a number of studies have reported that IGF-1 can mimic certain metabolic actions of insulin ([11] and references therein), there has been considerable controversy as to whether the metabolic effects of IGF-1 are exerted through IR or IGF-1R or through IR/IGF-1R hybrid receptors [12–14]. This issue was readdressed more recently with IR knockout mice. These mutants developed a severe diabetic ketoacidosis and hyperlipidemia associated with hepatic steatosis, and died within a week after birth [5,6]. Although IGF-1R cannot fully substitute for the absence of IR, there is now some evidence that certain metabolic effects could be achieved through IGF-1R in $IR^{-/-}$ mice. First, intraperitoneal injection of IGF-1 in $IR^{-/-}$ mice promptly reduced their hyperglycemia although plasma fatty acid levels were unaffected [15]. It was shown that IGF-1 treatment of $IR^{-/-}$ mice resulted in activation of phosphatidylinositol 3-kinase (PI 3-kinase) in skeletal muscle and liver and downregulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression in the liver indicating that the effect of IGF-1 in lowering blood glucose level was presumably due to the stimulation of peripheral glucose utilization and inhibition of hepatic gluconeogenesis, although these were not directly measured. Second, since $IR^{-/-}$ mice become hyperinsulinemic, one could wonder whether insulin, at high concentrations, could exert some of its effects through IGF-1R. This assumption was supported by the fact that double knockout mice for *Ins1* and *Ins2* develop the same metabolic disorders as $IR^{-/-}$ mice but more rapidly and die sooner [16].

In skeletal muscle, a major target tissue for the metabolic action of insulin in vivo, both IR and IGF-1R are present in differentiated muscle cells [17,18]. To further assess IGF-1R as an alternative receptor to IR for metabolic signaling, we have derived muscle cell lines from wild-type (wt) and $IR^{-/-}$ mice. We first examined the ability of wt and $IR^{-/-}$ myoblasts to differentiate into myotubes in culture. The results obtained indicated that IR is not required for this process. We could therefore perform a comparative analysis of the ability of IGF-1 and insulin to lead to metabolic effects and to activate signaling pathways in wt and $IR^{-/-}$ myotubes.

2. Materials and methods

2.1. Cell cultures

Skeletal muscle cell lines from wt and $IR^{-/-}$ newborn mice were obtained as described [19]. Briefly, posterior legs freed of skin and bones were minced and digested with trypsin and collagenase. After centrifugation, the cells were plated onto collagen-coated dishes and cultured in DMEM with 25 mM glucose/20% fetal calf serum (FCS)/2% ultroser (Biosepra). After few passages, stable muscle cell lines

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Abbreviations: IGF, insulin-like growth factor; IGF-1R, type 1 IGF-1 receptor; IR, insulin receptor; PI 3-kinase, phosphatidylinositol 3-kinase; MAP kinase, mitogen-activated protein kinase; IRS, insulin receptor substrate

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were recovered and cultured in the same medium. It was verified that the cell line from $IR^{-/-}$ mice carried a homozygous null mutation for IR by genotype analysis of cellular DNA [6]. All experiments were performed with cells between 20–40 passages. Confluent myoblast cultures differentiated into myotubes upon serum starvation (2–7% FCS) during 7–9 days.

2.2. RNA extraction and Northern blot analysis

Total RNA was extracted from cells using RNazol B (Campro). RNA samples (10 μg) were run on 1% formaldehyde-agarose gels and transferred onto nylon membranes. Filters were hybridized at 65°C with probes synthesized with cloned rat cDNA fragments specific for MyoD, myogenin and MLC1A as described [20] using a random priming kit (Amersham) and [α^{32}P]dCTP (3000 Ci/mmol; Amersham). The ribosomal S26 protein RNA was hybridized as internal control using a ^{32}P-labeled probe synthesized using a cloned cDNA fragment as indicated [20]. Following hybridization, the blots were washed and exposed to X-ray films for autoradiography for 1–2 weeks.

2.3. ^{125}I-IGF-1 binding assays

The binding of ^{125}I-IGF-1 was performed as follows. Myotubes were serum-starved for 18 h in DMEM with 5 mM glucose/0.2% bovine serum albumin (BSA), washed and incubated in the same medium for 2 h at 37°C. After washing with cold PBS, myotubes were incubated in the binding buffer (100 mM HEPES pH 7.6, 100 M NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 15 mM sodium acetate, 5 mM glucose, 1% BSA) containing ^{125}I-IGF-1 (25 000 cpm; 2000 Ci/mmol; Amersham). For non-specific binding, cold IGF-1 (GroPep) was added at 1 μM. After 4 h at 15°C, myotubes were washed with cold PBS and solubilized in 0.05% SDS/PBS at 37°C for 30 min and cell lysates were counted in a gamma counter. Protein concentrations were determined using the Bio-Rad reagent and BSA as standard.

2.4. 2-Deoxyglucose uptake

Myotubes were serum-starved for 18 h in DMEM with 5.5 mM glucose/0.1% BSA and then incubated for 2 h at 37°C in DMEM without glucose/25 mM HEPES. The myotubes were washed with PBS, stimulated with IGF-1 or insulin (Sigma) for 30 min at 37°C in HEPES buffer (50 mM HEPES pH 7.4, 140 mM NaCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$, 4.8 mM KCl)/0.1% BSA/0.1 mM 2-deoxyglucose and further incubated with 0.1 mM 2-deoxyglucose and 0.5 μCi of 2-deoxy-D-[2,6-^{3}H]glucose (25–55 Ci/mmol; Amersham) for 10 min at 37°C. Myotubes were washed and lysed as described [9] and the radioactivity was counted.

2.5. Glucose incorporation into glycogen

Glucose incorporation into glycogen was performed essentially as described [21]. After serum starvation as above, myotubes were incubated for 3 h at 37°C in DMEM/2.5 mM glucose/0.1% BSA/25 mM HEPES pH 7.6. After washing with PBS and stimulation with IGF-1 or insulin for 30 min at 37°C, 1 μCi of D-[U-^{14}C]glucose (250 mCi/mmol; Amersham) was added for 90 min. Myotubes were washed with cold PBS and solubilized with 30% KOH. After boiling the samples, glycogen was precipitated with 95% ethanol at 4°C and recovered on Whatman GFB filters which were counted for radioactivity.

2.6. IRS-1 phosphorylation

Myotubes were serum-starved for 18 h in DMEM/0.2% BSA, stimulated with IGF-1 or insulin for 3 min at 37°C and solubilized in the lysis buffer as described [9]. Lysates (0.3 mg) were incubated with anti-insulin receptor substrate-1 (IRS-1) rabbit polyclonal antibodies (Upstate Biotechnology) and protein A-Sepharose (Pharmacia) was added. The immunoprecipitates were run on 7.5% SDS–PAGE following electrotransfer onto nitrocellulose membranes (Schleicher and Schuell). Filters were blocked and first incubated with anti-IRS-1 rabbit polyclonal antibodies then with anti-rabbit horseradish peroxidase conjugated antibody (DAKO). Blots were revealed using the enhance chemiluminescence (ECL) detection system (Amersham) and exposed to X-ray films for 1–5 min. Membranes were then stripped and reblotted with anti-phosphotyrosine mouse monoclonal antibody (PY99; Santa Cruz).

2.7. Assay of PI 3-kinase activity

PI 3-kinase activity was measured as described [22]. Briefly, myotubes were serum-starved as above and stimulated with IGF-1 or insulin for 10 min at 37°C and solubilized in lysis buffer. Lysates (0.5 mg) were incubated with mouse monoclonal anti-phosphotyrosine antibody (PY20; Transduction Laboratories) and protein A-Sepharose. The pellets were washed and resuspended in 30 μl of the reaction buffer (20 mM HEPES pH 7.5, 0.4 mM EDTA and 0.4 mM $Na_4P_2O_7$) and 10 μl of L-α-phosphatidylinositol (10 mg/ml in 5 mM HEPES pH 7.5, Sigma). The reaction was started by adding 10 μl of a mixture containing 10 μCi of [γ-^{32}P]ATP (4500 Ci/mmol; ICN), 50 mM $MgCl_2$, 0.25 mM ATP, 14 mM HEPES pH 7.5, 0.28 mM EDTA and 0.28 mM $Na_4P_2O_7$ and shaken at room temperature for 15 min. The samples were processed and analyzed by thin layer chromatography as described [9] and the plates were exposed to X-ray films for 48 h.

2.8. Assay of MAP kinase phosphorylation

To examine the activation of the mitogen-activated protein kinase (MAP kinase), myotubes were serum-starved as above, stimulated with IGF-1 or insulin for 10 min at 37°C and solubilized in lysis buffer. Protein extracts (0.3 mg) were run on 12% SDS–PAGE and electrotransferred onto nitrocellulose membranes. Membranes were blocked and incubated with anti-active MAP kinase (p42/p44) specific rabbit polyclonal antibodies (Promega) and then treated with an anti-rabbit horseradish peroxidase conjugated secondary antibody. The filters were revealed using the ECL detection system and exposed to X-ray films for 2–5 min.

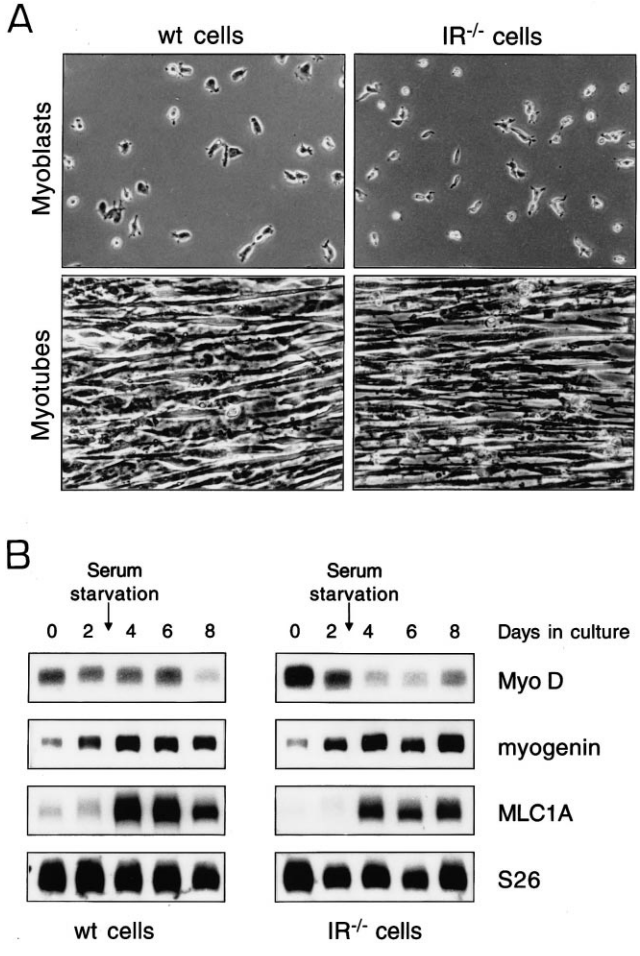

Fig. 1. Differentiation of wt and $IR^{-/-}$ muscle cell lines in culture. A: Morphology of wt and $IR^{-/-}$ muscle cells before and after fusion into myotubes. Magnifications are ×94 for myoblasts and ×188 for myotubes. B: Expression of muscle-specific genes during differentiation of wt and $IR^{-/-}$ cells. Subconfluent myoblasts (day 0) were grown to confluence and induced to differentiate by serum starvation at day 3. Total RNA was extracted and analyzed by Northern blot hybridization using ^{32}P-labeled probes as described in Section 2.

3. Results and discussion

It is now well established that IGF-1R plays an important role in cellular growth and differentiation [7]. However, the question as to whether IGF-1R has a full or limited metabolic potential in muscle, liver or fat tissue as compared to that of IR is still a matter of debate. In this work, we have derived muscle cell lines from wt and $IR^{-/-}$ mice to examine possible redundancy between IR and IGF-1R for metabolic action in muscle cells.

3.1. Differentiation of myoblasts into myotubes in the absence of IR

Both wt and $IR^{-/-}$ muscle cells presented the same aspect at the myoblast stage and were able to differentiate to form morphologically similar myotubes (Fig. 1A). Moreover, Troponin T, a marker specific for differentiated muscle cells, could be detected in wt as well as in $IR^{-/-}$ myotubes by immunocytochemistry using an anti-Troponin T antibody (not shown). Since muscle cell differentiation is accompanied by temporally regulated expression of genes encoding myogenic factors or muscle-specific structural proteins, we also examined the expression of such genes during the conversion of myoblasts into myotubes by Northern blot analysis. The expression of the MyoD gene appeared to be higher in $IR^{-/-}$ proliferating myoblasts as compared to wt myoblasts. Nevertheless, its expression decreased during the differentiation process for both lines (Fig. 1B). After inducing the differentiation of confluent myoblast cultures by serum starvation, the expression of genes for myogenin and MLC1A was increased within 24 h for both wt and $IR^{-/-}$ cells and was maintained at high level throughout the differentiation process (Fig. 1B). All these observations indicate that normal muscle cell differentiation can take place in the absence of IR.

To examine whether there exists any compensatory upregulation of IGF-1R in the absence of IR, we performed ^{125}I-IGF-1 binding experiments. Fig. 2 shows that ^{125}I-IGF-1 binding was very similar for both wt and $IR^{-/-}$ myotubes precluding any IGF-1R upregulation in $IR^{-/-}$ myotubes.

3.2. Metabolic action of IGF-1/insulin in $IR^{-/-}$ and wt myotubes

The ability of IGF-1 or insulin to stimulate glucose uptake and glucose incorporation into glycogen was measured in $IR^{-/-}$ and wt myotubes. Glucose transport was measured using the non-metabolized [^{3}H]-2-deoxyglucose analog. As shown in Fig. 3A, IGF-1 was able to stimulate glucose uptake in $IR^{-/-}$ myotubes and the dose–response curve was very similar to those obtained for wt myotubes with insulin or IGF-1. Insulin was also able to stimulate glucose uptake in $IR^{-/-}$ myotubes but the dose–response curve was shifted to the right due to the lower affinity of insulin for IGF-1R. For both wt and $IR^{-/-}$ myotubes, the maximal value for IGF-1 stimulated glucose uptake was comparable to that obtained at maximal insulin concentration.

Glucose incorporation into glycogen was measured using

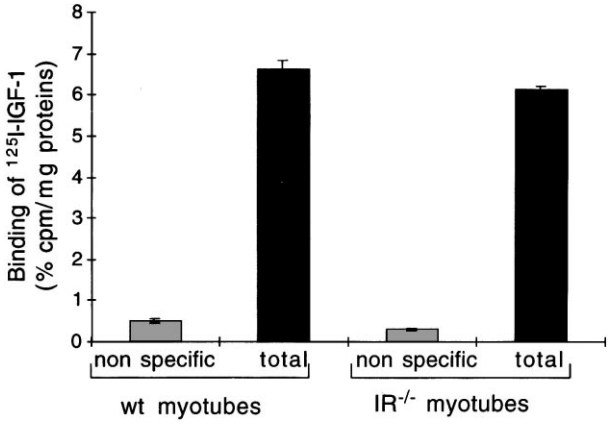

Fig. 2. Binding of ^{125}I-IGF-1 on wt and $IR^{-/-}$ myotubes. Non-specific binding corresponds to the values obtained in the presence of an excess of cold IGF-1. For details see Section 2. The results are means ± S.E.M. of three separate experiments.

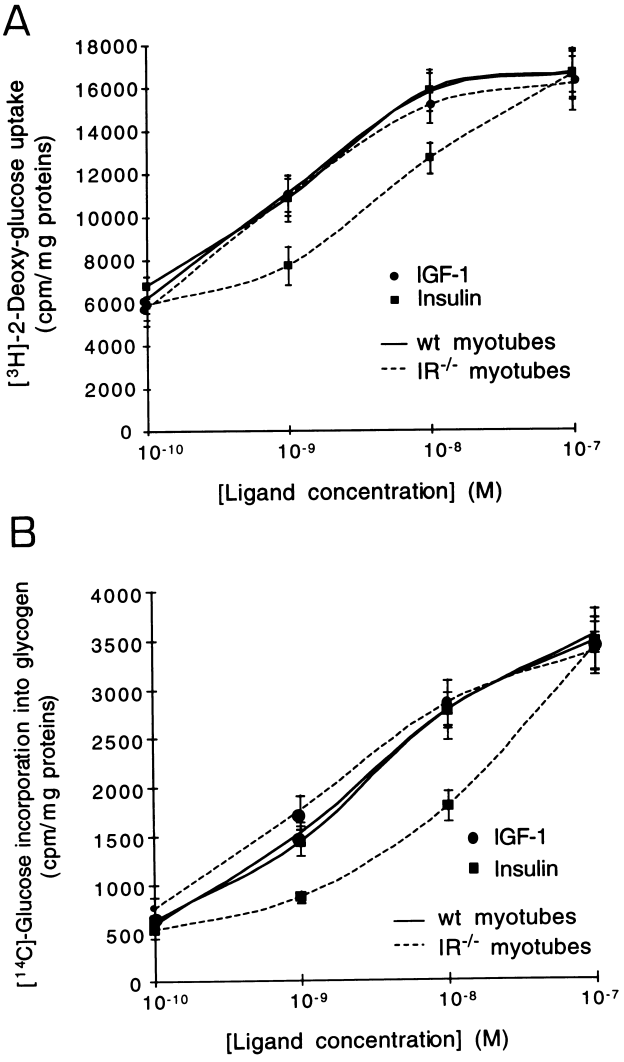

Fig. 3. Metabolic effects in wt and $IR^{-/-}$ myotubes stimulated by IGF-1 or insulin. A: Stimulation of glucose uptake. Cells were serum-starved, incubated in DMEM without glucose and stimulated with IGF-1 or insulin prior to incubation with [^{3}H]-2-deoxyglucose. The cells were washed, dissolved and the radioactivity was counted. A blank value obtained with unstimulated cells was subtracted. All data are means ± S.E.M. of 10 independent experiments. B: Stimulation of glucose incorporation into glycogen. Cells were serum-starved and stimulated with IGF-1 or insulin. After incubation with [^{14}C]glucose, cells were washed and solubilized. After boiling, glycogen was precipitated and recovered on filters which were counted for radioactivity. A blank value obtained with unstimulated cells was subtracted. All data are means ± S.E.M. of 10 independent experiments.

[^{14}C]glucose. As shown in Fig. 3B, IGF-1 could stimulate glucose incorporation into glycogen in IR$^{-/-}$ myotubes and the dose–response curve was very close to those obtained for wt myotubes with insulin or IGF-1. Again, glucose incorporation into glycogen in IR$^{-/-}$ myotubes was stimulated by insulin but the dose–response curve was shifted to the right as in the case of glucose uptake. The plateau values with IGF-1 or insulin for both lines were comparable.

These results indicate that IGF-1R can lead to metabolic effects in differentiated muscle cells with an efficiency that is comparable to that of IR. Moreover, insulin, at higher concentrations, could lead to metabolic effects through IGF-1R.

3.3. Signaling pathways activated by IGF-1/insulin in IR$^{-/-}$ and wt myotubes

The mechanisms of signal transduction by IR and IGF-1R consist in tyrosine phosphorylation of intermediate docking proteins such as IRS-1, -2, -3 and -4 which subsequently recruit various effector proteins. This, in turn, results in the activation of signaling pathways such as the PI 3-kinase or the MAP kinase pathways [3]. Since IRS-1 appears to be the predominant signaling intermediate in muscle cells [23], we examined IRS-1 phosphorylation as well as PI 3-kinase and MAP kinase activation in IR$^{-/-}$ and wt myotubes in response to IGF-1 or insulin.

To measure IRS-1 phosphorylation, lysates from myotubes stimulated with IGF-1 or insulin were immunoprecipitated with anti-IRS-1 antibodies and analyzed by Western blotting using anti-IRS-1 or anti-phosphotyrosine antibodies. Fig. 4A shows that both IGF-1 and insulin can induce IRS-1 phosphorylation in wt as well as in IR$^{-/-}$ myotubes. As expected, IRS-1 phosphorylation in IR$^{-/-}$ myotubes by insulin required higher concentrations. Attempts to detect IRS-2 phosphorylation in wt or IR$^{-/-}$ myotubes in response to IGF-1 or insulin were not successful.

To examine PI 3-kinase activation, lysates from myotubes stimulated with IGF-1 or insulin were immunoprecipitated using anti-phosphotyrosine antibodies. The PI 3-kinase activity in immunoprecipitates was measured by phosphorylation of phosphoinositols in the presence of [γ-^{32}P]ATP and analyzing the products by chromatography. The autoradiograms presented in Fig. 4B show that PI 3-kinase was activated in IR$^{-/-}$ and wt myotubes by IGF-1 or insulin. Again, activation of PI 3-kinase by insulin in IR$^{-/-}$ myotubes was observed with higher concentrations. Interestingly, PI 3-kinase activation in wt myotubes was found to be more efficient with IGF-1 than with insulin.

Finally, stimulation of MAP kinase in IR$^{-/-}$ and wt myotubes in response to IGF-1 or insulin was examined in cell lysates by Western immunoblotting using an anti-active MAP kinase antibody which specifically recognizes phosphorylated MAP kinase (p42/p44). As shown in Fig. 4C, MAP kinase

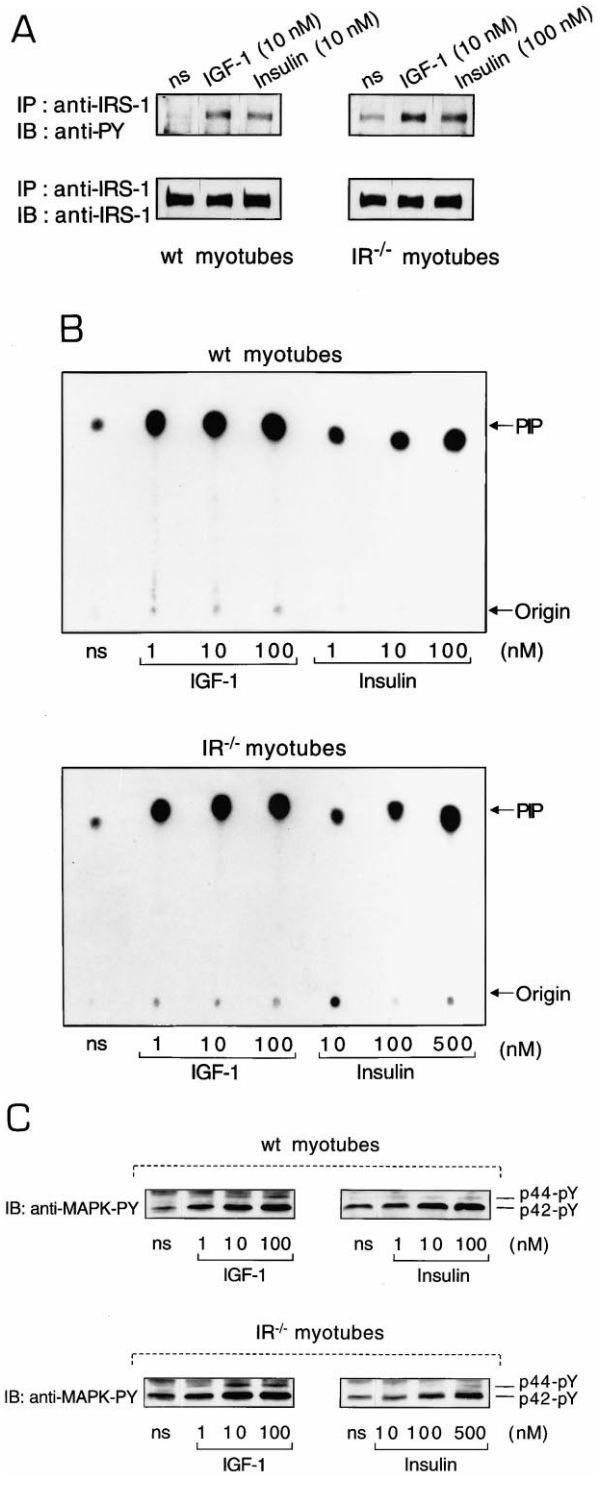

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Fig. 4. Activation of signaling pathways in wt and IR$^{-/-}$ myotubes stimulated by IGF-1 or insulin. A: Induction of IRS-1 phosphorylation. Cell lysates from wt and IR$^{-/-}$ myotubes stimulated with IGF-1 or insulin were immunoprecipitated with anti-IRS-1 antibodies and immunocomplexes were analyzed by Western blotting using anti-IRS-1 antibodies. The filters were then stripped and reblotted with anti-phosphotyrosine (PY) antibodies. Blots were revealed using the ECL detection system. B: Stimulation of PI 3-kinase activity. Cell lysates from wt and IR$^{-/-}$ myotubes stimulated with IGF-1 or insulin were immunoprecipitated with anti-PY antibodies and PI 3-kinase activity was measured by phosphorylation of phospholipids in the presence of [γ-^{32}P]ATP. The products were analyzed by thin layer chromatography and the autoradiogram is presented. C: Activation of MAP kinase. Cell lysates from wt and IR$^{-/-}$ myotubes stimulated with IGF-1 or insulin were run on 12% SDS–PAGE and transferred onto nitrocellulose filters. Immunoblotting was performed using an anti-active MAP kinase antibody. Blots were revealed using the ECL detection system. ns: non-stimulated. These results are representative of two separate experiments for IRS-1 phosphorylation and three independent experiments for PI 3-kinase and MAP kinase assays.

was activated in wt and $IR^{-/-}$ myotubes stimulated with insulin or IGF-1, although basal MAP kinase activity was not completely suppressed in serum-starved cells. Higher insulin concentrations were required for MAP kinase activation in $IR^{-/-}$ myotubes. For both lines, phosphorylation of p42 was more pronounced than that of p44.

In conclusion, IGF-1 as well as insulin at higher concentration can activate signaling pathways in $IR^{-/-}$ myotubes. These effects are comparable to those obtained upon stimulating wt myotubes with insulin or IGF-1.

3.4. IGF-1R as an alternative receptor for metabolic signaling in vivo?

The present study clearly shows that IGF-1R can represent an alternative receptor to IR for metabolic signaling in $IR^{-/-}$ myotubes in culture. Thus, IGF-1R-mediated stimulation of glucose uptake and utilization in skeletal muscle could partly account for the hypoglycemic effects of IGF-1 injection in $IR^{-/-}$ mice [15]. Mutant mice in which muscle-specific IR knockout (MIRKO) was achieved using the Cre-loxP system represent another unique in vivo situation to address the question of the metabolic potential of IGF-1R [24]. MIRKO mice were able to maintain euglycemia for several months with normal plasma insulin. The compromised insulin signaling in skeletal muscle can thus be perfectly compensated in these mutants. Interestingly, MIRKO mice presented increased fat deposits in several sites suggesting that glucose was likely being shunted from muscle to fat tissue or liver. Since IGF-1R was not found to be upregulated and little or no IGF-1R or IRS-1 phosphorylation was observed in response to insulin in the skeletal muscle of MIRKO mice, the issue of whether IGF-1R could function as an alternative receptor to IR was not further explored. In light of the present work, it would be interesting to reexamine the ability of IGF-1 to lead to some metabolic effects through IGF-1R in isolated skeletal muscle or in vivo using MIRKO mice.

The ability of IGF-1R to lead to metabolic effects in the liver was also examined recently using a hepatocyte cell line derived from $IR^{-/-}$ mice. It was shown that IGF-1R activation does not enhance glycogen synthesis and fails to inhibit glucose production by these cells [10]. Thus, IGF-1 action through IGF-1R in the liver probably does not contribute much to the hypoglycemic effect of IGF-1 injection into $IR^{-/-}$ mice. The conclusion that IGF-1R could not efficiently substitute for the absence of IR in the liver also emerged from the analysis of liver-specific IR knockout (LIRKO) mice which presented dramatic insulin resistance, severe glucose intolerance and a failure of insulin to suppress hepatic glucose production and to regulate hepatic gene expression [23].

Finally, the question of whether metabolic signaling can be achieved through IGF-1R in fat tissue cannot be easily addressed in $IR^{-/-}$ mice since this tissue is very underdeveloped in these mutants [25]. Further work would be required to investigate whether the failure of IGF-1 to reduce circulating free fatty acid levels in $IR^{-/-}$ mice results from diminished fat tissue in these mutants or reflects a lower capacity of IGF-1R to lead to metabolic effects of IR on lipogenesis/lipolysis.

In conclusion, IGF-1R could represent an alternative receptor to IR for metabolic signaling at least in the skeletal muscle. However, it appears from all the recent studies performed using global and/or tissue-specific knockout of genes encoding IR or insulin that the metabolic effects through IGF-1R, in vivo, would be achieved only under certain pathophysiological conditions in which the ligands, IGFs or insulin, become available in excess.

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