Differential Signaling Activation by Insulin and Insulin-Like Growth Factors I and II upon Binding to Insulin Receptor Isoform A

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A variety of human malignancies overexpresses isoform A of the insulin receptor (IR-A) and produces IGFs (IGF-I and/or IGF-II). IR-A binds IGF-II with high affinity (although 4-fold lower than that for insulin), whereas it binds IGF-I with low affinity (approximately 30-fold lower than that for insulin). However, in engineered cells expressing only the IR-A, but not IGF-I receptor (R^-/IR-A cells), IGF-II is a more potent mitogen than insulin. Herein, we investigated downstream signaling of IGF-II, IGF-I, and insulin in R^-/IR-A cells to better understand their role in cell growth. We found that despite inducing a lower IR-A autophosphorylation than insulin, IGF-II was more potent than insulin for activating p70S6 kinase (p70S6K) and approximately equally potent in activating the early peaks of ERK1/2 and Akt. However, ERK1/2 activation persisted longer after IGF-II, whereas Akt activation persisted longer after insulin. Therefore, cells stimulated with IGF-II had a higher p70S6K/Akt activation ratio than cells stimulated with insulin. Remarkably, IGF-I also elicited a similar signaling pattern as IGF-II, despite inducing minimal IR-A autophosphorylation. ERK1/2 and protein kinase C seem to be involved in the preferential stimulation of p70S6K by IGFs. In conclusion, our study has identified a novel complex role of IR-A, which not only elicits a unique signaling pattern after IGF-II binding but also induces substantial downstream signaling upon binding to the low-affinity ligand IGF-I. These results underline the role of IR-A in physiology and disease. (***Endocrinology*** **150: 3594–3602, 2009)**

Insulin receptor (IR) isoform A (IR-A), one of the two isoforms of the IR, is generated by the skipping of exon 11 of the IR gene and differs from the other IR isoform (IR-B) because it lacks a stretch of 12 amino acid residues at the carboxy terminus of the IR α-subunit (1–3). Both IR isoforms are coexpressed in cells, and the relative abundance of IR-A and IR-B is regulated by development stage- and tissue-specific factors (4, 5). IR-A is predominantly expressed in fetal and cancer cells, whereas IR-B is predominantly expressed in differentiated insulin target cells (6, 7). Unlike IR-B, which is a highly specific receptor for insulin, IR-A exhibits high affinity for insulin, intermediate affinity for IGF-II, and low affinity for IGF-I (6). IGF-II binds to IGF type I receptor (IGF-IR) and to IR-A with similar affinities and shares with the homolog IGF-I potent mitogenic and antiapoptotic effects (6, 8). Despite the homology with IGF-II, IGF-I binds with high affinity only to the IGF-IR, and its affinity for the IR-A is approximately 10-fold lower than that of IGF-II (6) although approximately 3-fold higher than its affinity for the IR-B. At present, the activation of intracellular signaling upon IGF-I binding to IR is considered negligible, although a recent study has reported that IGF-I may elicit biological effects by binding, but not phosphorylating, both IR-A and IR-B (9).

In contrast, several studies have firmly established that IGF-II elicits biological effects via IR-A. For instance, in mouse fibroblasts expressing only IR-A and not IGF-IR (R^-/IR-A cells), IGF-II was a more potent mitogen than insulin itself (6). In SKUT-1 human rhabdomyosarcoma cells, which lack functional IGF-IR and express almost only IR-A, IGF-II was more potent than insulin in inducing cell chemotaxis (10). Moreover, the global profile of gene expression elicited by IGF-II and insulin in

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Abbreviations: BIM, Bisindolylmaleimide I; 4EBP1, 4E-binding protein 1; IGF-IR, IGF type I receptor; IR, insulin receptor; IR-A, IR isoform A; IRS, IR substrate; JNK, c-Jun N-terminal protein kinase; MEK1, MAPK kinase 1; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent kinase-1; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; p70S6K, p70S6 kinase.

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R^-/IR-A is partially different (11), with IGF-II being more potent than insulin in regulating certain genes (11). However, the signaling pathways responsible for this unique biological response after IR-A activation by IGF-II are not completely understood, although there is evidence that IGF-II, compared with insulin, induces a different balance between the phosphatidylinositol 3-kinase (PI3-K) and the ERK1/2 pathways (6, 10). The strong signaling activation and biological effects elicited by IGF-II binding to IR-A, despite a relatively low receptor autophosphorylation, led us to hypothesize that also the low-affinity ligand IGF-I could elicit downstream signaling by binding to IR-A.

We therefore investigated the activation of the downstream signaling in R^-/IR-A cells after stimulation with IGF-II, IGF-I, or insulin, with a focus on kinase p70S6 kinase (p70S6K), a serine/threonine protein kinase, which is activated by several growth factors and plays a crucial role in cell growth and proliferation (12–14). Activation of p70S6K depends on the sequential phosphorylation of serine/threonine sites; full p70S6K activation requires the 3-phosphoinositide-dependent kinase-1 (PDK1) and the Raptor/mammalian target of rapamycin (mTOR) complex, both located downstream of PI3-K (15, 16). However, other kinases, such as ERK1/2, may also affect p70S6K activation (17).

We found that IGF-II induces a higher p70S6K/Akt ratio than insulin and that also the low-affinity ligand IGF-I stimulates a similar intracellular signaling than IGF-II.

Materials and Methods

Materials

See supplemental material (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Cell cultures

R^- mouse fibroblasts (kindly provided by Renato Baserga, Philadelphia, PA) are mouse 3T3-like cells derived from animals with a targeted disruption of the IGF-IR gene. R^- cells, which express low endogenous IR (approximately 5×10^3 receptors per cell) (18), were cotransfected with the pNTK2 expression vector containing the cDNA for the human IR-A ($Ex11^-$) and with the pPDV61 plasmid encoding the puromicin resistance gene, by using the Lipofectamine reagent (Life Technologies, Inc./BRL, Bethesda, MD), as previously described (6). Cell clones obtained (R^-/IR-A cells) expressed approximately 3×10^5 to 5×10^5 receptors per cell (19).

Western blotting analysis

Subconfluent cells were serum starved for 24 h and then treated with insulin, IGF-II, or IGF-I at the indicated doses at 37 C. Ligand stimulation was terminated, and cells were solubilized as previously described (19). Whole-cell lysates were then subjected to reducing SDS-PAGE on 10% polyacrylamide gel. The resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis with the indicated antibodies. All immunoblots were revealed by the enhanced chemiluminescence method (Amersham, Little Chalfont, UK), autoradiographed, and subjected to densitometric analysis.

RT-PCR

RT-PCR for mouse IR isoforms was carried out as previously described (20) with oligonucleotide primers spanning nucleotides 2147–2171 (5′-ATC AGA GTG AGT ATG ACG ACT CGG-3′) and 2349–2373 (5′-TCC TGA CTT GTG GGC ACA ATG GTA-3′) of the mouse IR. PCR amplification was carried out for 30 cycles of 30 sec at 94 C, 30 sec at 60 C, and 45 sec at 72 C in a DNA thermal cycler 9700 (Applied Biosystems Inc., Foster City, CA). After electrophoresis of the PCR products, the 286- and 250-bp DNA fragments representing $Ex11^+$ and $Ex11^-$ IR isoforms were analyzed by scanning densitometry.

Statistical analysis

Phosphorylation curves in response to either insulin or IGF-II were compared by two-way ANOVA. Differences between means were analyzed by the Student's t test for paired samples. A P value <0.05 was considered statistically significant. Statistical analysis was carried out with GraphPad software (Prism, London, UK).

Results

Activation kinetics of p70S6K, 4E-binding protein 1 (4EBP1), and IR-A by IGF-II and insulin

Activation of the p70S6K is a crucial step for both cell survival and growth in response to IGFs and insulin (12, 21). Therefore, we first compared the activation of p70SK in response to IGF-II and insulin. After phosphorylation of Thr-421 and Ser-424, full activation of p70S6K requires phosphorylation of Thr-389, which is dependent on the Raptor/mTOR complex (22), is strictly related to the p70S6K activity, and is a prerequisite for the phosphorylation of Thr-229 by PDK1 (23).

Time-course of p70S6K phosphorylation

In cells incubated with both IGF-II and insulin (10 nM), Thr-421/Ser-424 phosphorylation started within 5 min and reached a peak at 40 min, remaining elevated from 20–80 min. At 180 min, Thr-421/Ser-424 phosphorylation declined but was still well above baseline levels (Fig. 1A). Thr-389 phosphorylation followed a similar pattern, except for showing a more evident and earlier decline, especially after insulin (Fig. 1B). On the average, IGF-II was significantly more potent than insulin in inducing p70S6K phosphorylation at Thr-389 ($P = 0.005$), whereas differences observed with Thr-421/Ser-424 phosphorylation did not reach statistical significance ($P = 0.09$) (Fig. 1, A and B).

Time course of 4EBP1 phosphorylation

The elongating factor 4EBP1 is also phosphorylated by the Raptor/mTOR complex. 4EBP1 phosphorylation requires two steps. In the first step, mTOR phosphorylates Thr-37 and Thr-46. The second phosphorylation step involves serum-dependent phosphorylation at Ser-65 and Thr-70. We studied the phosphorylation pattern of 4EBP1 with a phosphoantibody recognizing the Thr-37/Thr-46 phosphosite. After both insulin and IGF-II, 4EBP1 phosphorylation reached high levels at 20 min. It tended to decline at 180 min after IGF-II but not after insulin. On the average, insulin was more potent than IGF-II ($P = 0.008$) (Fig. 1C). Very similar results were obtained with phosphorylation on Thr-70 (not shown).

These data indicate that IGF-II potently activates p70S6K and 4EBP1 phosphorylation by binding to the IR-A. However, p70S6K was predominantly phosphorylated by IGF-II, whereas 4EBP1 was predominantly phosphorylated by insulin.

Time course of IR-A autophosphorylation

The higher p70S6K phosphorylation observed after IGF-II compared with insulin apparently contrasts with the lower IGF-II binding affinity to IR-A (6). We therefore evaluated the time course of IR-A autophosphorylation (Tyr-1150/1151) after IGF-II and insulin. IR-A autophosphorylation after IGF-II was 50–60% that observed after insulin in terms of maximum values and remained significantly lower than after insulin at all time points ($P = 0.0001$) (Fig. 1D).

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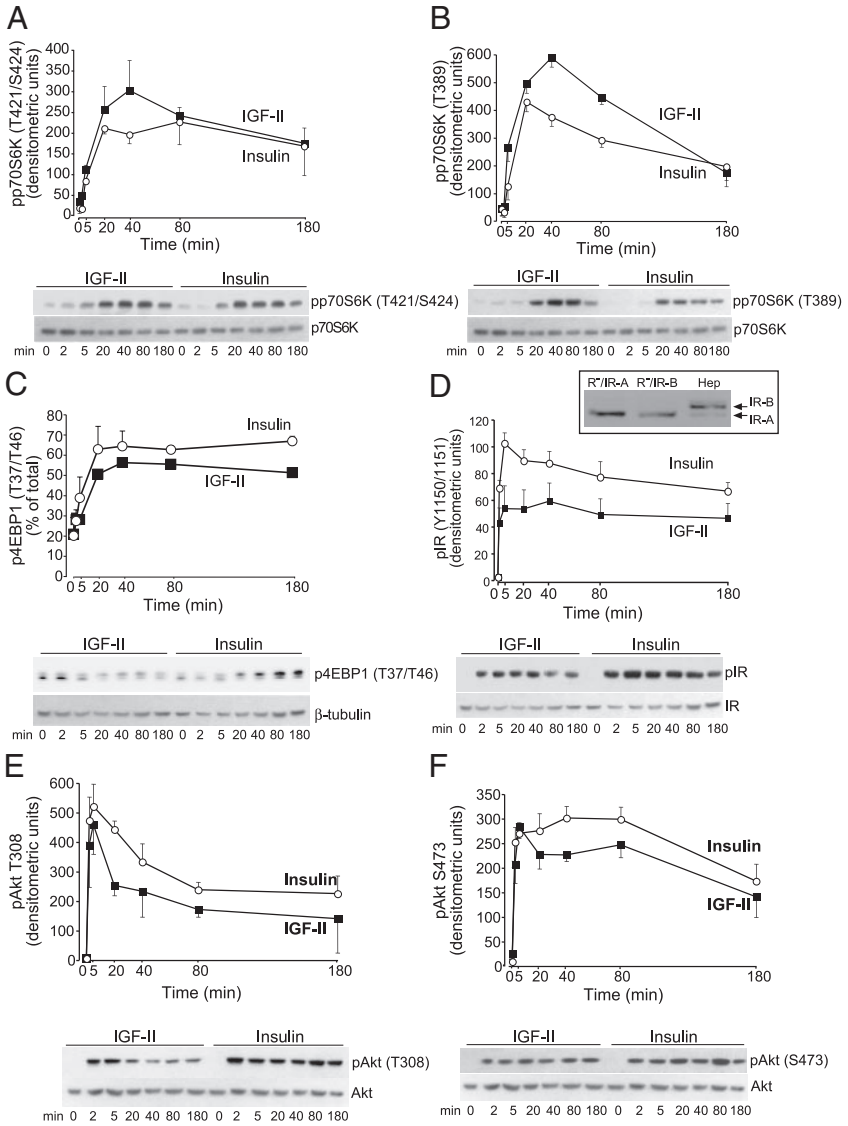

FIG. 1. Time course of IGF-II- and insulin-mediated phosphorylation of p70S6K, 4EBP1, IR, and Akt in R⁻/IR-A cells. R⁻/IR-A cells were serum starved for 24 h. After stimulation with 10 nM IGF-II or insulin for the indicated times, cells were lysed and analyzed by Western blot. Filters were immunoblotted with phospho-T421/S424-p70S6K antibody (A), phospho-T389-p70S6K antibody (B); phospho-T37/T46-p4EBP1 antibody (C), and phospho-Y1150/Y1151-IR (D). The *top panels* show the mean and SE of four separate experiments, and the *bottom panels* show a representative experiment. The same blots were probed with an anti-p70S6K or an anti-IR or an anti-β-tubulin antibody to check for protein loading. Values of T389-p70S6K phosphorylation after IGF-II were significantly higher than after insulin ($P = 0.005$). However, the proportion of T37/T46-p4EBP1 was higher after insulin than after IGF-II ($P = 0.009$). D, *Inset* shows endogenous (mouse) IR isoforms expressed in IR-A or IR-B transfected R⁻ cells (R⁻/IR-A and R⁻/IR-B) and in control mouse hepatocytes (Hep), as determined by RT-PCR. E and F, R⁻/IR-A cells were serum starved for 24 h and stimulated with 10 nM IGF-II or insulin for the indicated times. Cells were then lysed and analyzed by SDS-PAGE and immunoblotted with either phospho-T308-Akt (E) or phospho-S473-Akt (F) antibodies. The *top panels* show the mean and SE of three separate experiments, and the *bottom panels* show a representative experiment of three. The same blots were probed with an anti-Akt or an anti-β-tubulin antibody to check for protein loading. Values of Akt phosphorylation were significantly higher after insulin than after IGF-II ($P = 0.034$ for Ser473-Akt and $P = 0.036$ for Thr308-Akt).

R⁻/IR-A cells express low levels of endogenous (mouse) IRs (60- to 100-fold lower than the transfected human IR-A). Although these mouse IRs should not interfere with the response to the transfected IR-A, we evaluated the relative abundance of mouse IR isoforms in R⁻/IR-A cells. We found that R⁻/IR-A cells express exclusively the mouse IR-A (Fig. 1D, *inset*) as well as R⁻ cells transfected with the human IR-B (R⁻/IR-B). In contrast, mouse hepatocytes express predominantly the mouse IR-B.

IGF-II, via the IR-A receptor, induces a higher p70S6K/Akt activation ratio than insulin

Similarly to p70S6K, full Akt activation requires both PI3-K and mTOR. PI3-K is needed because Akt translocates to the cell membrane, where it interacts with phosphoinositols produced by PI3-K activity and then is phosphorylated by PDK1 at Tyr-308. mTOR is needed because it phosphorylates Akt on Ser-473 after interacting with Rictor and forming a Rictor/mTOR complex (24). We therefore evaluated whether the kinetics of Akt phosphorylation at both sites was similar to p70S6K phosphorylation. Phosphorylation on Thr-308 peaked at 2–5 min with both ligands and then sharply declined, with insulin being slightly more potent (Fig. 1E). Phosphorylation on Ser-473 also markedly increased at 2–5 min with both IGF-II and insulin. However, Ser-473 phosphorylation remained high until 80 min, with insulin being more potent than IGF-II (Fig. 1F). On average, insulin was significantly more potent than IGF-II both on Ser-473-Akt ($P = 0.034$) and on Thr-308-Akt ($P = 0.036$). Therefore, the p70S6K/Akt activation ratio was higher after IGF-II than after insulin (2.72 ± 0.12 *vs.* 1.25 ± 0.13, respectively at 40 min, $P = 0.02$).

Three Akt isoforms (Akt1 to Akt3) have been described, with different tissue-specific expression and functional characteristics. We therefore asked whether a differential Akt isoforms activation by IGF-II and insulin could account for the partially different biological effects of IGF-II and insulin. R⁻/IR-A cells were incubated in the presence or absence of IGF-II or insulin at 10 nM for 30 min. IGF-I was also used. Cell lysates were immunoprecipitated with isoform-specific antibodies to Akt and then blotted with non-isoform-specific phosphoantibodies to Akt. We found that Akt1 is the predominant isoform in R⁻/IR-A cells and accounted for most of the phosphorylation signal observed on Ser-473 and virtually the entire phosphorylation on Thr-308 (Fig. 2C). Although both Akt2 and Akt3 showed low ligand-stimulated Ser-473 phosphorylation, they did not show any phosphorylation on Thr-308 (expect for a barely detectable signal after insulin) (Fig. 2C). Insulin was the most potent stimulus for all Akt isoforms (supplemental Fig. 1).

IGF-II, via the IR-A receptor, induces a higher ERK1/2/Akt activation ratio than insulin

We next evaluated the activation kinetics of the various MAPKs in response to IGF-II and insulin. ERK1/2 phosphorylation activation was biphasic after both IGF-II and insulin. The first activation peak was evident at 2–5 min and the second one much later (80 min) (Fig. 2A). On average, the intensity of the first peak was slightly more elevated with insulin, whereas the second peak was slightly more elevated and prolonged with IGF-II (Fig. 2A). The differences between the two ligands, however, did not reach statistical significance. A similar kinetics was also observed for c-Jun N-terminal protein kinase (JNK) phosphorylation; insulin tended to be more potent than IGF-II at early time points (2–20 min) but slightly less potent at late time points (80–180 min) (Fig. 2B). p38 MAPK phosphorylation was also biphasic,

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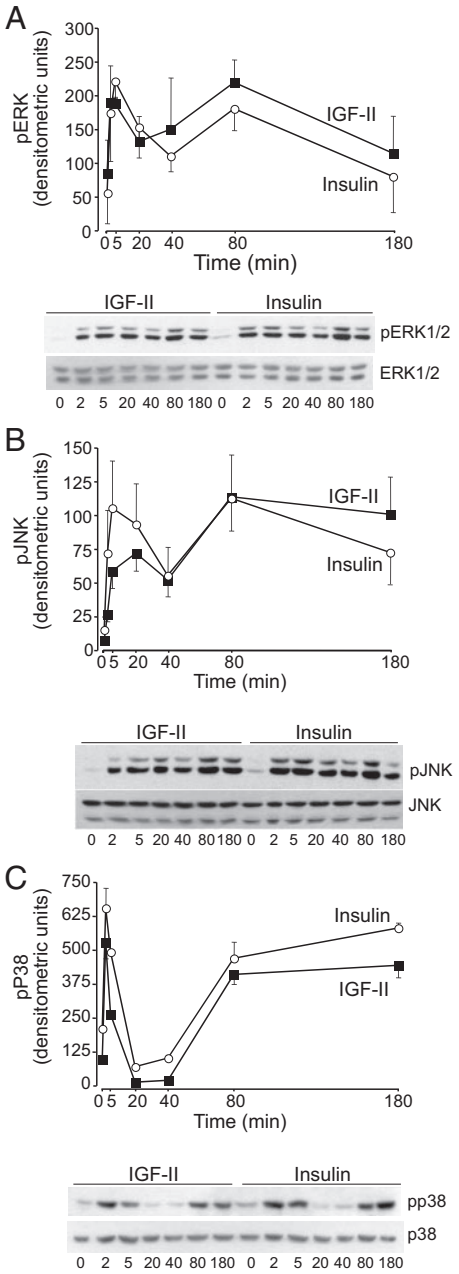

FIG. 2. Time course of ERK1/2, JNK, and p38 phosphorylation in response to IGF-II and insulin. R^-/IR-A cells were serum starved for 24 h and stimulated with 10 nM IGF-II or insulin for the indicated times. Cells were then lysed and analyzed by SDS-PAGE and immunoblotted with phospho-ERK1/2 antibody (A), phospho-JNK antibody (B), and phospho-p38 antibody (C). The *top panels* show the mean and SE of three separate experiments, and the *bottom panels* show a representative experiment of three. The same blots were probed with an anti-ERK1/2, an anti-JNK, or an anti-p38 antibody to check for protein loading. Two-way ANOVA showed no significant difference between the two ligands with regard to ERK1/2 and JNK activation curves. However, insulin was more potent than IGF-II in eliciting p38 phosphorylation ($P = 0.0001$).

with an early peak at 2–5 min and a late peak at 80–180 min. Insulin was more potent than IGF-II at all time points ($P = 0.0001$) (Fig. 2C). When analyzed together with Akt data, these findings indicate that IGF-II and insulin differentially affect the balance between the active forms of ERK1/2 and Akt. As a consequence, a higher ERK1/2/Akt activation ratio is seen after IGF-II than after insulin, especially at the late time points (0.92 ± 0.17 *vs.* 0.53 ± 0.10 at 80 min, for IGF-II and insulin, respectively, $P = 0.043$).

IGF-I induces downstream signaling via IR-A

The high p70S6K/Akt and ERK1/2/Akt activation ratios caused by cell exposure to IGF-II occurred at a lower IR-A phosphorylation than that induced by insulin. We wondered, therefore, whether also the low-affinity IR-A ligand IGF-I could elicit similar effects and whether this effect was dose dependent. Dose-response experiments were carried out using IGF-I, IGF-II, or insulin. After exposure to insulin, IR-A phosphorylation became detectable at 0.1 nM and reached the maximum level at 10 nM. As expected, IGF-II and IGF-I were weaker stimulators of IR-A phosphorylation, being approximately 10- and 100-fold less effective than insulin, respectively (Fig. 3A, *top*).

Akt phosphorylation (on both Thr-308 and Ser-473) followed a similar pattern, although it was more marked than expected in cells stimulated with IGF-I. In contrast, p70S6K phosphorylation followed a different pattern; it was already clearly stimulated at 0.1 nM with all three ligands and reached the maximum at 1.0 nM after IGF-II and insulin (Fig. 3A, *top*). There was no clear difference between the phosphorylation pattern on Thr-389 and on Thr-424/Ser-421, although phosphorylation on Thr-389 was more marked. The ratio between p70S6K phosphorylation (Thr-389) and Akt phosphorylation (Ser-473), was, therefore, significantly higher ($P < 0.05$) with IGFs than with insulin, especially at low ligand concentrations (Fig. 3A, *bottom*). ERK1/2 phosphorylation was less pronounced than Akt or p70S6K phosphorylation and often peaked at 0.1–1.0 nM after IGF-II and insulin (Fig. 4A, *top*). mTOR-dependent 4EBP1 phosphorylation, was, however, slightly more marked with insulin than with IGFs (Fig. 3A).

Time-course experiments with each ligand at 10 nM confirmed that both IGF-I and IGF-II induce a higher p70S6K/Akt activation ratio than insulin, especially at late time points (20–80 min) (Fig. 3B, *top* and *bottom*).

Because p70S6K and ERK1/2 activation occurred with IGF-I concentrations that induce minimal IR-A autophosphorylation, we evaluated whether these effects are really dependent on IR-A autophosphorylation and do not involve nonspecific interactions of IGFs with other membrane receptors. Indeed, cell preincubation with the IR tyrosine kinase inhibitor hydroxy-2-naphthalenylmethylphosphonic acid (0.5 μM) switched off the phosphorylation of downstream kinases (p70S6K, Akt, and ERK1/2) induced by insulin and by IGFs, confirming that these effects are dependent on IR-A autophosphorylation (Fig. 3C). In contrast, cell incubation with an epidermal growth factor receptor tyrosine kinase inhibitor (AG1478, 20 μM) did not affect IGFs or insulin signaling (Fig. 3C).

IR substrate (IRS)-1 and IRS-2 phosphorylation: possible role in the pp70S6/pAkt balance after IGFs and insulin

To identify possible differences in intracellular signaling explaining the different p70S6K/Akt activation ratio observed after IGFs and insulin, we evaluated the relative potency of the three ligands in eliciting phosphorylation of immediate IR substrates IRS-1 and IRS-2.

IRS-1 tyrosine phosphorylation was parallel to the degree of IR-A phosphorylation (Fig. 4A). In contrast, IRS-2 tyrosine phosphorylation was equally activated by IGF-II and insulin (Fig. 4A).

IRS-1 protein may also be phosphorylated on several serine residues that are involved in a negative feedback loop that limits the effects of IR stimulation. We therefore evaluated IRS-1 phosphorylation on Ser-636/Ser-639, which is p70S6K/mTOR dependent, and on Ser-307, which is phosphorylated by JNK but also by other kinases including p70S6K. The degree of IRS-1 serine phosphorylation after IGFs was not significantly different from that reached after insulin (Fig. 4, B and C) despite the markedly lower IR phosphorylation obtained with IGFs as compared with insulin. The high levels of IRS-1 serine phosphorylation were in close agreement with the high p70S6K activation observed after IGFs.

ERK1/2 may have a role in the differential activation of p70S6K by IGFs and insulin

PI3-K-dependent activation of the Raptor/mTOR complex is a critical step for full p70S6K activation. However, other kinases, such as the

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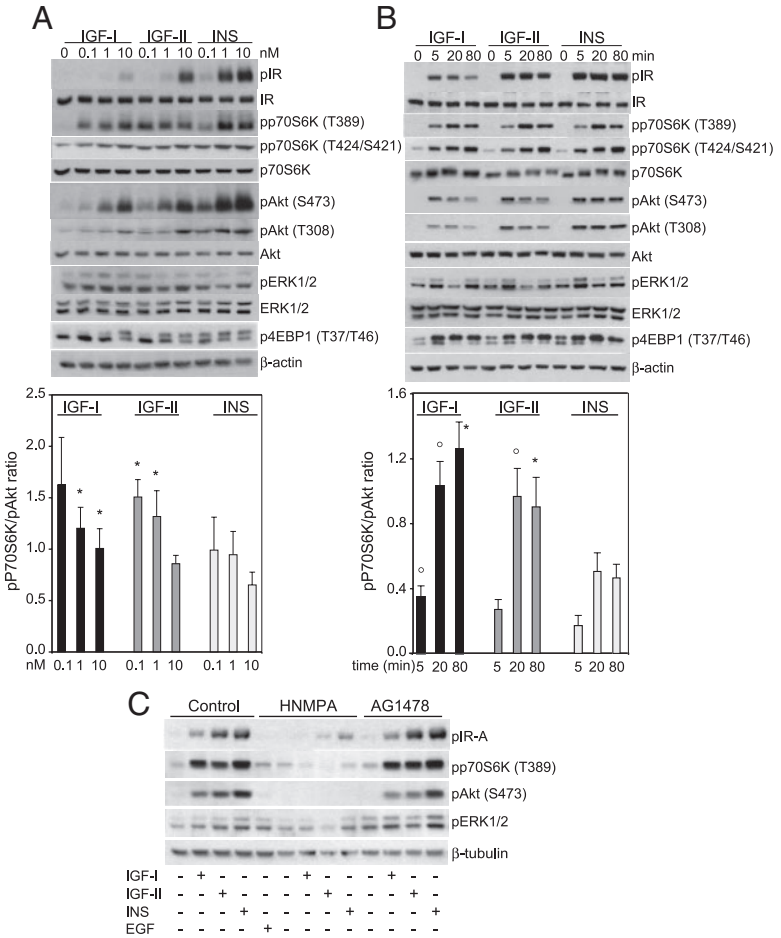

FIG. 3. Time course and dose response of IR phosphorylation and downstream signaling after stimulation with IGF-I, IGF-II, and insulin. Effect of IR tyrosine kinase inhibition. A, R$^-$/IR-A cells were serum starved for 24 h and then exposed to the indicated concentrations of either IGF-I or IGF-II or insulin, solubilized, and analyzed by Western blot. Filters were probed with phospho-antibodies to pIR (Y1150/Y1151), p70S6K (T421/S424-p70S6K and T389-p70S6K), pAkt (T308-Akt and S473-Akt), phospho-ERK1/2 (T202/Y204), and phospho-4EBP1 (T37/T46). Immunoblotting with anti-IR, anti-p70S6K, anti-Akt, and anti-β-actin antibodies was used to check for protein loading. The *top panel* shows a representative experiment of three. The *bottom panel* shows the p70S6K/Akt activation ratio (mean ± SE) obtained from three experiments. *, $P < 0.05$, IGF-I *vs.* insulin and IGF-II *vs.* insulin. B, R$^-$/IR-A cells were incubated with 10 nM of either IGF-I or IGF-II or insulin and processed for Western blot analysis at the indicated time points, as above. Filters were probed as above. The *top panel* shows a representative experiment of three. The *bottom panel* shows the p70S6K/Akt activation ratio (mean ± SE) obtained from three experiments. *, $P < 0.05$; °, $P < 0.03$, IGF-I and IGF-II *vs.* insulin. C, R$^-$/IR-A cells were serum starved for 24 h and preincubated for 60 min with either the IR inhibitor hydroxy-2-naphthalenylmethylphosphonic acid (0.5 μM) or the epidermal growth factor (EGF) receptor inhibitor AG1478 (20 μM) as negative control. Cells were then exposed to 10 nM IGF-I, IGF-II, or insulin, solubilized, and analyzed by Western blot. Filters were probed with phospho-antibodies to p70S6K (T389–p70S6K), pAkt (S473-Akt), and phospho-ERK1/2. Immunoblotting with anti-β-tubulin antibody was used to check for protein loading. IR inhibition, but not epidermal growth factor receptor inhibition, completely blocked downstream signaling by all three ligands.

MAPKs, may also play a role. In particular, ERK1/2 may be involved in p70S6K phosphorylation on Thr-421 and Ser-424. We therefore evaluated the possible role of these kinases in the differential p70S6K activation by IGFs and insulin. R$^-$/IR-A cells were first preincubated with either rapamycin (10 nM, inhibitor of the Raptor/mTOR complex) or with LY294002 (5 μM, inhibitor of PI3-K) or with PD98059 or U0126 [50 and 5 μM, respectively, both inhibitors of the MAPK kinase 1 (MEK1)/ERK1/2 pathway] and then exposed to IGF-I, IGF-II, or insulin (10 nM for 20 min).

Rapamycin virtually abolished p70S6K phosphorylation at Thr-389 with no difference among the three ligands (Fig. 5A). Moreover, rapamycin slightly increased Akt phosphorylation at Ser-473 ($P = 0.05$) in response to all three ligands, but especially to IGFs (Fig. 5A), as expected by the blockade of feedback inhibition of IRS-1 by the mTOR/p70S6K pathway.

The PI3-K inhibitor LY294002 completely abolished ligand-stimulated p70S6K on Thr-389 and markedly reduced phosphorylation on Thr-421/Ser-424. LY294002 also abolished Akt phosphorylation (Fig. 5B).

Cell incubation with the MEK1 inhibitors PD98059 or U0126 markedly inhibited ligand-stimulated ERK1/2 phosphorylation and decreased p70S6K phosphorylation, especially after IGFs. Akt phosphorylation on Ser-473 remained unaltered or slightly increased (Fig. 5B). ERK1/2 inhibition, therefore, shifted the p70S6K/Akt activation ratio in favor of Akt. This effect was more evident in cells stimulated with IGFs ($P = 0.005$) rather than with insulin (Fig. 5C). These data suggest that the ERK1/2 pathway contributes to the p70S6K activation especially in response to IGFs.

The possible role of other members of the MAPK family was evaluated by preincubating cells with SP600125, a specific inhibitor of JNK, or with SB203580, an inhibitor of p38 MAPK. p70S6K phosphorylation was not affected by SP600125 (not shown), whereas it was partially inhibited by SB203580, which also blunted the Akt response (not shown).

Possible role of protein kinase C (PKC) in affecting the p70S6K and Akt phosphorylation balance after IGFs and insulin

The PKC pathway may also be involved in p70S6K regulation. Several PKC isoforms are stimulated by insulin/IGFs. In particular, the PKCδ, belonging to novel PKCs, and PKCζ, belonging to atypical PKCs, have been involved in the regulation of p70S6K activity in response to insulin/IGFs.

We first evaluated the effect of rottlerin, a PKCδ inhibitor, and of P9103-71, a PKCζ inhibitor. Preincubation with rottlerin did not elicit any significant effect on ligand-stimulated p70S6K phosphorylation (not shown). In contrast, preincubation with P9103-71 slightly reduced p70S6K phosphorylation but slightly increased S473-Akt phosphorylation (Fig. 6A). The net effect was a reduced p70S6K/Akt activation ratio. However, although this trend was consistent, the effect did not reach statistical significance (Fig. 6B).

We then evaluated the effect of a more general PKC inhibitor, GF109203X, also termed bisindolylmaleimide I (BIM). BIM has been reported to specifically inhibit classical and novel PCK isoforms at 1 μM whereas it also inhibits atypical PKCs at 10 μM. Preincubation with 1 μM BIM did not affect p70S6K phosphorylation in response to either IGFs or insulin (Fig. 6C). However, it slightly but consistently increased Akt-Ser473 phosphorylation in response to IGF-II and insulin (+18.3 ± 4%, $P = 0.04$) but not to IGF-I. ERK1/2 phosphorylation was unaffected. Preincubation with 10 μM BIM slightly increased Thr-389-p70S6K phosphorylation in response to IGF-II and insulin (+15.2 ± 3% and +26.2 ± 4%, respectively, not significant) but not in response to IGF-I (+3.1 ± 4%) (Fig. 6C). ERK1/2 phosphorylation similarly increased in cells stimulated with IGF-II or insulin but not with IGF-I. Ser-473-Akt phosphorylation increased in response to all three ligands (+21 ± 15%, +35 ± 9%, and +55 ± 11% after IGF-I, IGF-II, and insulin, respectively), although it reached statistical significance only for IGF-II and insulin ($P < 0.05$) (Fig. 6C). As a consequence, preincubation with BIM tended to decrease the p70S6K/Akt phosphorylation ratio. This difference reached significance for IGFs after 10 μM BIM ($P = 0.045$ and $P = 0.003$, after IGF-I and IGF-II, respectively) (Fig. 6D).

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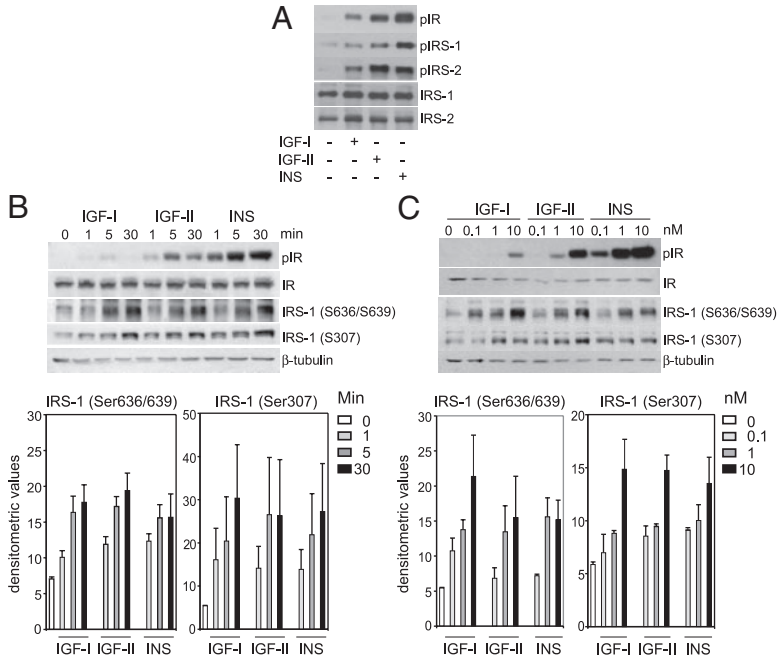

FIG. 4. IRS protein phosphorylation in response to IGF-I, IGF-II, and insulin. A, IR, IRS-1, and IRS-2 tyrosine phosphorylation in R$^-$/IR-A cells incubated with IGF-I, IGF-II, or insulin (10 nM for 5 min). Cell lysates were immunoprecipitated with anti-IR, anti-IRS-1, or anti-IRS-2 and then immunoblotted with anti-Py. Immunoblotting with anti-IRS-1 and anti-IRS-2 antibodies were used to control for protein loading. B and C, Dose response (B) and time course (C) of phosphorylation of IR (Y1150/Y1151) and serine IRS-1 (residues S636/S639 and S307) in cells treated with IGF-I, IGF-II, and insulin for 30 min. Immunoblotting with anti-IR and anti-β-tubulin antibodies was used to control for protein loading. In B and C, *bottom panels* represent composite graphs of IRS-1 serine phosphorylation. *Columns* represent means of three separate experiments; *bars* are SE.

These data suggest that PKCs have a complex role in the balance of p70S6K and Akt phosphorylation. PKCs, especially atypical PKCs, may have a direct stimulatory effect on p70S6K activity both after insulin and IGFs. However, the increase of Akt and p70S6K phosphorylation after BIM confirms that PKCs are also involved in the inhibitory feedback of IR-A signaling, especially after insulin or IGF-II stimulation, whereas IGF-I is less active.

Discussion

In the present study, we investigated the pattern of intracellular signaling elicited by both IGF-I and IGF-II in cells null for the IGF-IR and overexpressing the IR isoform A (R$^-$/IR-A cells). We found that not only IGF-II but also IGF-I elicits a peculiar signaling pattern characterized by a relatively high p70S6K response, compared with insulin. More specifically, major results of our study can be summarized as follows: 1) IGF-II activates p70S6K and ERK1/2 at levels similar or even higher than insulin despite inducing approximately 50% lower IR-A autophosphorylation than insulin, 2) IGF-II induces a higher p70S6K/Akt activation ratio compared with insulin, and 3) IGF-I elicits a similar signaling pattern than IGF-II, although it binds IR-A with much lower affinity than IGF-II.

These differences in postreceptor signaling between IR-A ligands may account for the more potent mitogenic effect but less potent metabolic effect of IGF-II in respect to insulin in R$^-$/IR-A cells (6, 25) and for the partially different patterns of gene expression after IGF-II and insulin (26).

Of special relevance is the observation that IGF-I may elicit high p70S6K activation in R$^-$/IR-A cells despite causing very low IR-A phosphorylation. The IGF-I binding affinity for the IR-A is approximately 10-fold lower than that for IGF-II and approximately 30-fold lower than that of insulin. Nevertheless, this IGF-I effect requires IR-A phosphorylation, because a specific IR phosphorylation inhibitor blocks it. It is worth noting that endogenous (mouse) IRs present in R$^-$/IR-A cells cannot influence the signaling differences observed because they are expressed as IR-A themselves.

Our present data are in agreement with recent data of Denley *et al.* (9), who reported that IGF-I might induce biological effects in R$^-$ cells expressing either IR-A or IR-B. These authors, however, observed only a delayed low-level Akt activation in cells exposed to IGF-I; p70S6K was not investigated (9). In our study, IGF-I elicited a clear Akt phosphorylation although less pronounced than p70S6K phosphorylation.

The mechanisms through which both IGFs cause similar or even higher p70S6K activation than insulin, despite a lower IR-A phosphorylation, appear complex and dose dependent. p70S6K is activated by different kinases at multiple sites (22, 27). Initial phosphorylation involving the autoinhibitory pseudosubstrate domain (Ser-411, Ser-418, Thr-421, Ser-424, and Ser-429) and the catalytic domain extension (Thr-390 and Ser-394) relieves pseudosubstrate suppression at the autoinhibitory domain, allowing phosphorylation on Thr-389 and Thr-229 in the catalytic domain and consequent full activation (22, 27) by the Raptor/mTOR complex and the PDK1, respectively (15, 23, 28, 29).

Akt activation requires first Ser-473 phosphorylation by the mTOR/Rictor complex (24) followed by Thr-308 phosphorylation by PDK1 (15). In our system, although IGF-II and insulin were approximately equipotent in terms of Akt peak stimulation, the IGF-II effect was more transient. This caused a higher p70S6K/Akt activation ratio than insulin, especially at 20–80 min. IGF-I was an even weaker Akt stimulator than IGF-II.

Akt exists in three isoforms (30). Because Akt1 has been predominantly implicated in growth stimulation but Akt2 in metabolic effects, we evaluated whether IGFs and insulin differentially activate Akt isoforms. We found that Akt1 was the most represented isoform in R$^-$/IR-A cells, with Akt2 and Akt3 being expressed at a much lower level. Akt1 accounted for most Akt phosphorylation, and no Akt isoform appeared preferentially activated by IGFs. Therefore, the differential biological effects elicited by insulin and by IGF-II in R$^-$/IR-A cells (6, 25) are not likely to be mediated by a differential activation of Akt isoforms.

Another interesting feature of IR-A signaling is that both IGFs appear to shift the balance of phosphorylation from IRS-1 to IRS-2. These data are in agreement with our previous results obtained with IGF-II (6). Whether and how this could be related with the shift in the balance of p70S6K/Akt activation is at the moment unclear.

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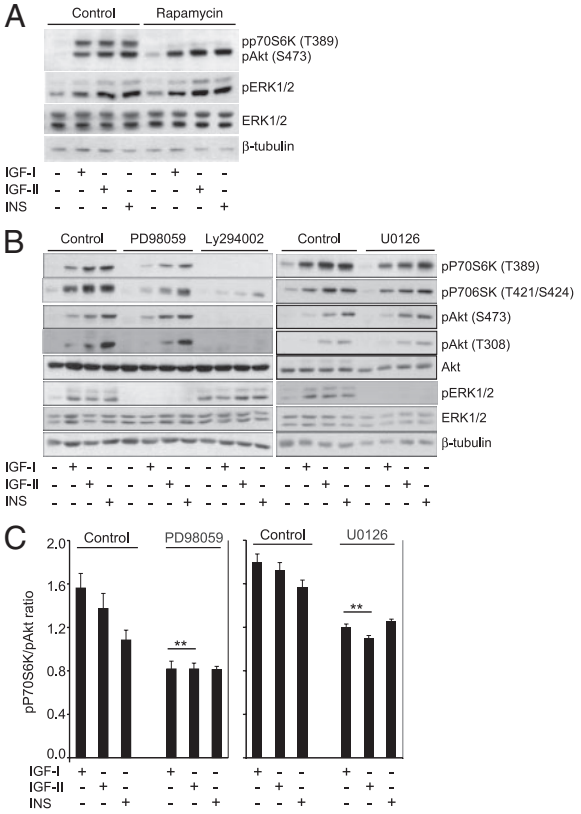

FIG. 5. Effect of inhibitors of mTOR, MEK, and PI3-K. Serum-starved R$^-$/IR-A cells were preincubated for 60 min with the Raptor/mTOR complex inhibitor rapamycin (10 nM) (A) or the MEK inhibitors PD098059 (50 μM) and U0126 (5 μM) or the PI3-K inhibitor Ly294002 (10 μM) (B). Cells were then exposed to 10 nM of IGF-I, IGF-II, or insulin, solubilized, and analyzed by Western blot. Filters were probed as indicated. Immunoblotting with anti-Akt and/or anti-ERK and anti-β-tubulin antibodies was used to check for protein loading. A representative experiment is shown. C, p70S6K/Akt activation ratio in cells preincubated or not with PD98059 (mean ± SE of four experiments) or U0126 (mean ± SE of three experiments). Data showed a significant shift in the p70S6K/Akt phosphorylation balance in response to IGF-I and IGF-II in cells preincubated with both inhibitors ($P < 0.01$).

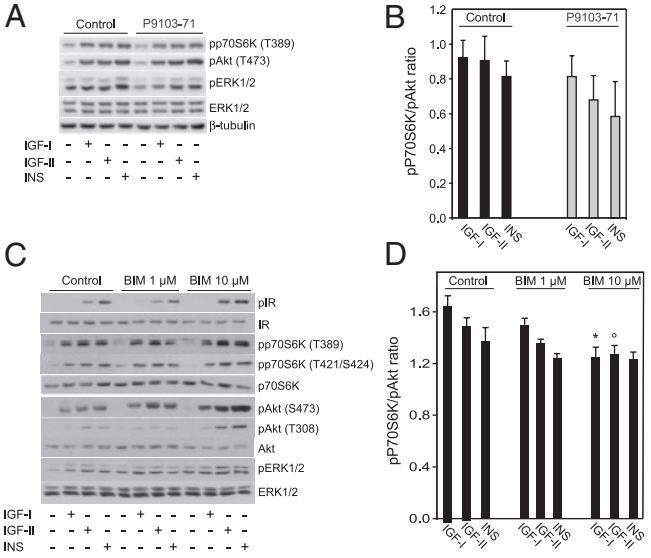

FIG. 6. Effect of PKC inhibitors. Serum-starved R$^-$/IR-A cells were preincubated for 60 min with either the PKCζ inhibitor, PKCζ myristoylated pseudosubstrate (P9103-71, 5 μM) (A and B) or with the broad-range PKC inhibitor BIM (1 and 10 μM) (C and D). Cells were then exposed to 10 nM IGF-I, IGF-II, or insulin for 20 min, solubilized, and analyzed by Western blot. Filters were probed as indicated. A representative of three different experiments is shown. B and D, p70S6K/Akt activation ratio (mean ± SE of three experiments) in cells preincubated or not with either P9103-71 (B) or BIM (D). *, $P = 0.045$; °, $P = 0.003$, BIM *vs.* control.

However, this different balance of p70S6K/Akt activation may in turn differentially affect IRS-1 phosphorylation on serine residues, a mechanism that provides a negative feedback loop and contributes to fine-tune the inhibition of insulin/IGF stimulation (31). In fact, the high IGF-mediated p70S6K activation was mirrored by IRS-1 phosphorylation on Ser-636/Ser-639 and Ser-307 (32). Specifically, Ser-636/Ser-639 phosphorylation, which interferes with the PI3-K/Akt signaling (33), is mTOR dependent (34) and also requires p70S6K1 (32). Ser-307 phosphorylation, which promotes a general inhibition of IRS-1 signaling (35), requires both the PI3-K/mTOR/p70S6K cascade and JNK (36). We found that IRS-1 phosphorylation on Ser-636/Ser-639 and Ser-307 was approximately equally stimulated by IGFs and insulin.

Inhibition of Raptor/mTOR complex by rapamycin completely inhibited p70S6K phosphorylation after IGFs or insulin, indicating that Raptor/mTOR activity is equally required for p70S6K activation by all three ligands. However, 4EBP1 phosphorylation, also downstream of the Raptor/mTOR complex (37, 38), was greater after insulin than after IGFs, being, therefore, partially dissociated from p70S6K activation. These data are in line with previous studies carried out in different model systems (14, 39, 40).

PI3-K inhibition by LY294002 abolished Thr-389-p70S6K phosphorylation and markedly reduced Thr-421/Ser-424-p70S6K phosphorylation in cells stimulated with either IGFs or insulin, indicating that PI3-K activity is required for full p70S6K activation by all three ligands. LY294002 also inhibited Akt phosphorylation on both Thr-308 and Ser-473, as expected.

Kinases other than Raptor/mTOR and PI3-K are involved in the regulation of p70S6K activity (41–43). The residues Thr-421/Ser-424 in the autoinhibitory domain are located in a canonical MAPK phosphorylation site (41), and both JNK and ERK1/2 may affect p70S6K activation (42, 44). We examined, therefore, the possible role of members of the MAPK family. IGFs elicited a higher ERK1/2/Akt activation ratio than insulin, especially at low ligand concentrations. In fact, although Akt phosphorylation reached the maximum at 10 nM ligand concentration, both p70S6K and ERK1/2 appear to require a lower IR-A activation threshold. ERK1/2 inhibition by PD98059 or U0126 reduced p70S6K phosphorylation while leaving unaffected or slightly enhancing Akt phosphorylation, especially after IGFs. Taken together, these findings suggest that ERK1/2 may contribute to the preferential activation of p70S6K by IGFs.

PKC isoforms appear to play a complex role in the balance of p70S6K and Akt phosphorylation in response to insulin and IGFs. The PKC family includes several isoforms that can be grouped into three categories: conventional, novel, and atypical (45). The activity of several PKC isoforms may be regulated by insulin and IGFs (46). In turn, components of all three PCK categories play a dual role by stimulating some branches of insulin signaling, such as p70S6K activity, and feeding back to inhibit insulin/IGF signaling by inducing serine IRS phosphory-

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lation (46). We evaluated the possible role of PKCζ, an atypical PKC (47) and of PKCδ, a novel PKC. PKCζ is activated by PDK1 with a PI3-K-dependent mechanism in response to insulin (48). Although it may phosphorylate p70S6K in certain model systems (49), PKCζ may also bind directly to Akt reducing its downstream signaling (50). We found that inhibition of PKCζ slightly reduced p70S6K phosphorylation with some increase of Akt phosphorylation.

Although PKCδ has also been involved in p70S6K phosphorylation by insulin (45), the inhibition of PKCδ was ineffective in our system. PKC inhibition by the bisindolylmaleimide derivative BIM markedly increased Akt phosphorylation in response to IGF-II and to insulin while only slightly increasing p70S6K. This is consistent with the release of PKC-dependent negative feedback on Akt phosphorylation. The lower/absent increase of p70S6K phosphorylation is consistent with the blockade of the dual effects of PKCs, which can both stimulate p70S6K directly and inhibit it by feeding back al the level of IRSs (46, 51). Interestingly, these data suggest that the limited ability of IGF-I to activate this PKC-dependent negative feedback plays a role in causing unexpectedly high p70S6K phosphorylation.

In conclusion, novel and unexpected findings indicate that, besides IGF-II, also IGF-I can induce significant downstream signaling via IR-A despite low-affinity binding and minimal IR-A autophosphorylation. Unique features of both IGF-I and IGF-II signaling in IGF-IR null cells expressing only IR-A are a high p70S6K/Akt and ERK1/2/Akt activation ratios. These findings may partially account for the different biological effects of IGF-II and insulin observed in these cells and add new complexity to the IGF system. Moreover, these results foster our understanding on the complex role of IR-A overexpression in cancer and strongly support the concept that IR-A should be regarded as a target for anticancer therapies.

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References

1. **Seino S, Bell GI** 1989 Alternative splicing of human insulin receptor messenger RNA. Biochem Biophys Res Commun 159:312–316
2. **Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A, McClain DA** 1990 Functionally distinct insulin receptors generated by tissue-specific alternative splicing. EMBO J 9:2409–2413
3. **Yamaguchi Y, Flier JS, Benecke H, Ransil BJ, Moller DE** 1993 Ligand-binding properties of the two isoforms of the human insulin receptor. Endocrinology 132:1132–1138
4. **Kosaki A, Webster NJ** 1993 Effect of dexamethasone on the alternative splicing of the insulin receptor mRNA and insulin action in HepG2 hepatoma cells. J Biol Chem 268:21990–21996
5. **Webster NJ, Kong Y, Sharma P, Haas M, Sukumar S, Seely BL** 1997 Differential effects of Wilms tumor WT1 splice variants on the insulin receptor promoter. Biochem Mol Med 62:139–150
6. **Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, Goldfine ID, Belfiore A, Vigneri R** 1999 Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 19:3278–3288
7. **Belfiore A** 2007 The role of insulin receptor isoforms and hybrid insulin/IGF-I receptors in human cancer. Curr Pharm Des 13:671–686
8. **Krywicki RF, Yee D** 1992 The insulin-like growth factor family of ligands, receptors, and binding proteins. Breast Cancer Res Treat 22:7–19
9. **Denley A, Carroll JM, Brierley GV, Cosgrove L, Wallace J, Forbes B, Roberts Jr CT** 2007 Differential activation of insulin receptor substrates 1 and 2 by insulin-like growth factor-activated insulin receptors. Mol Cell Biol 27:3569–3577
10. **Sciacca L, Mineo R, Pandini G, Murabito A, Vigneri R, Belfiore A** 2002 In IGF-I receptor-deficient leiomyosarcoma cells autocrine IGF-II induces cell invasion and protection from apoptosis via the insulin receptor isoform A. Oncogene 21:8240–8250
11. **Pandini G, Medico E, Conte E, Sciacca L, Vigneri R, Belfiore A** 2003 Differential gene expression induced by insulin and insulin-like growth factor-II through the insulin receptor isoform A. J Biol Chem 278:42178–42189
12. **Volarevic S, Thomas G** 2001 Role of S6 phosphorylation and S6 kinase in cell growth. Prog Nucleic Acids Res Mol Biol 65:101–127
13. **Fingar DC, Salama S, Tsou C, Harlow E, Blenis J** 2002 Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev 16:1472–1487
14. **Fingar DC, Richardson CJ, Tee AR, Cheatham L, Tsou C, Blenis J** 2004 mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4EBP1/eukaryotic translation initiation factor 4E. Mol Cell Biol 24:200–216
15. **Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, Ashworth A, Bownes M** 1997 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. Curr Biol 7:776–789
16. **Sarbassov DD, Ali SM, Sabatini DM** 2005 Growing roles for the mTOR pathway. Curr Opin Cell Biol 17:596–603
17. **Iijima Y, Laser M, Shiraishi H, Willey CD, Sundaravadivel B, Xu L, McDermott PJ, Kuppuswamy D** 2002 c-Raf/MEK/ERK pathway controls protein kinase C-mediated p70S6K activation in adult cardiac muscle cells. J Biol Chem 277:23065–23075
18. **Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, Rubin R, Efstratiadis A, Baserga R** 1994 Effect of a null mutation of the insulin-like growth factor I receptor gene on growth and transformation of mouse embryo fibroblasts. Mol Cell Biol 14:3604–3612
19. **Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A** 2002 Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem 277:39684–39695
20. **Entingh AJ, Taniguchi CM, Kahn CR** 2003 Bi-directional regulation of brown fat adipogenesis by the insulin receptor. J Biol Chem 278:33377–33383
21. **Pullen N, Thomas G** 1997 The modular phosphorylation and activation of p70s6k. FEBS Lett 410:78–82
22. **Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J** 1998 Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. J Biol Chem 273:16621–16629
23. **Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR** 1994 Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol Cell Biol 14:4902–4911
24. **Sarbassov DD, Guertin DA, Ali SM, Sabatini DM** 2005 Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098–1101
25. **Morrione A, Valentinis B, Xu SQ, Yumet G, Louvi A, Efstratiadis A, Baserga R** 1997 Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc Natl Acad Sci USA 94:3777–3782
26. **Pandini G, Conte E, Medico E, Sciacca L, Vigneri R, Belfiore A** 2004 IGF-II binding to insulin receptor isoform a induces a partially different gene expression profile from insulin binding. Ann NY Acad Sci 1028:450–456
27. **Moser BA, Dennis PB, Pullen N, Pearson RB, Williamson NA, Wettenhall RE,**

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Kozma SC, Thomas G 1997 Dual requirement for a newly identified phosphorylation site in p70s6k. Mol Cell Biol 17:5648–5655
28. **Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, Thomas G** 1995 The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. EMBO J 14:5279–5287
29. **Burnett PE, Barrow RK, Cohen NA, Snyder SH, Sabatini DM** 1998 RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4EBP1. Proc Natl Acad Sci USA 95:1432–1437
30. **Fayard E, Tintignac LA, Baudry A, Hemmings BA** 2005 Protein kinase B/Akt at a glance. J Cell Sci 118:5675–5678
31. **Zick Y** 2005 Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. Sci STKE 2005:pe4
32. **Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G** 2004 Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 431:200–205
33. **Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A** 2005 Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology 146:1328–1337
34. **Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB** 2001 A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci USA 98:4640–4645
35. **Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF** 2002 Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem 277:1531–1537
36. **Müssig K, Staiger H, Fiedler H, Moeschel K, Beck A, Kellerer M, Häring HU** 2005 Shp2 is required for protein kinase C-dependent phosphorylation of serine 307 in insulin receptor substrate-1. J Biol Chem 280:32693–32699
37. **Sonenberg N, Pause A** 2006 Signal transduction. Protein synthesis and oncogenesis meet again. Science 314:428–429
38. **Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K** 2002 Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell 110:177–189
39. **Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J** 1998 Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem 273:14484–14494
40. **Wang X, Beugnet A, Murakami M, Yamanaka S, Proud CG** 2005 Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol Cell Biol 25:2558–2572
41. **Mukhopadhyay NK, Price DJ, Kyriakis JM, Pelech S, Sanghera J, Avruch J** 1992 An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. J Biol Chem 267:3325–3335
42. **Lehman JA, Gomez-Cambronero J** 2002 Molecular crosstalk between p70S6k and MAPK cell signaling pathways. Biochem Biophys Res Commun 293:463–469
43. **Wang X, Proud CG** 1997 p70 S6 kinase is activated by sodium arsenite in adult rat cardiomyocytes: roles for phosphatidylinositol 3-kinase and p38 MAP kinase. Biochem Biophys Res Commun 238:207–212
44. **Cano E, Hazzalin CA, Mahadevan LC** 1994 Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. Mol Cell Biol 14:7352–7362
45. **Murphy LO, Blenis J** 2006 MAPK signal specificity: the right place at the right time. Trends Biochem Sci 31:268–275
46. **Moschella PC, Rao VU, McDermott PJ, Kuppuswamy D** 2007 Regulation of mTOR and S6K1 activation by the nPKC isoforms, PKCε and PKCδ, in adult cardiac muscle cells. J Mol Cell Cardiol 43:754–766
47. **Sampson SR, Cooper DR** 2006 Specific protein kinase C isoforms as transducers and modulators of insulin signaling. Mol Genet Metab 89:32–47
48. **Hirai T, Chida K** 2003 Protein kinase Cζ (PKCζ): activation mechanisms and cellular functions. J Biochem 133:1–7
49. **Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A** 1998 Regulation of protein kinase Cζ by PI 3-kinase and PDK1. Curr Biol 8:1069–1077
50. **Romanelli A, Martin KA, Toker A, Blenis J** 1999 p70 S6 kinase is regulated by protein kinase Cζ and participates in a phosphoinositide 3-kinase-regulated signalling complex. Mol Cell Biol 19:2921–2928
51. **Doornbos RP, Theelen M, van der Hoeven PC, van Blitterswijk WJ, Verkleij AJ, van Bergen en Henegouwen PM** 1999 Protein kinase Cζ is a negative regulator of protein kinase B activity. J Biol Chem 274:8589–8596

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