Insulin and Interleukin-4 Induce Desensitization to the Mitogenic Effects of Insulin-like Growth Factor-I

PIVOTAL ROLE FOR INSULIN RECEPTOR SUBSTRATE-2*

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Insulin-induced desensitization to insulin-like growth factor-I (IGF-I) stimulated mitogenesis in bovine fibroblasts involves steps distal to IGF-I binding to its tyrosine kinase receptor. When quiescent cultures of bovine fibroblasts were stimulated with 10 nM IGF-I and total cell lysates immunoblotted with anti-phosphotyrosine antibody, we observed a band at ~97 kDa, representing the β-subunit of the IGF-I receptor, and a predominant tyrosyl-phosphorylated species migrating as a broad band between 170 and 190 kDa. The majority of proteins in this latter band were immunoprecipitated by antibodies against insulin receptor substrate (IRS)-2 and not by antibodies against IRS-1. Pretreatment of bovine fibroblasts with 10 nM insulin for 48 h blocked subsequent IGF-I-stimulated DNA synthesis and the IGF-I-induced increase in tyrosyl-phosphorylated IRS-2. Insulin pretreatment did not alter IRS-1 or IRS-2 expression by these cells, as assessed by metabolic labeling and direct immunoblotting with IRS antibodies. The interleukin-4 (IL-4) cytokine receptor also has IRS-2 as its major substrate for tyrosine phosphorylation. Although 10 nM IL-4 was as effective as 10 nM IGF-I in stimulating IRS-2 phosphorylation, 10 nM IL-4 did not have comparable mitogenic power in these cells. Nonetheless, pretreatment of bovine fibroblasts with IL-4 inhibited IGF-I-stimulated DNA synthesis by 50–60%, concomitant with a decrease in IGF-I-induced IRS-2 phosphorylation. Insulin-induced desensitization could be prevented if a specific inhibitor of phosphatidylinositol 3-kinase (LY294002), but not an inhibitor of mitogen-activated protein kinase (PD98059), was present during the preincubation period. LY294002 also prevented the shift in IRS-2 molecular mass in response to prolonged incubation of cells with insulin. These data indicate that, in a nontransformed cell system, IRS-2 plays a key role in cellular desensitization to IGF-I-stimulated mitogenesis most likely through a feedback mechanism in the phosphatidylinositol 3-kinase pathway. Furthermore, they suggest that signaling through IRS-2 may provide an important point of integration for hormone, growth factor, and cytokine receptor systems that regulate critical cellular growth responses.

Peptide growth factors bind to specific transmembrane receptors to initiate intracellular events that must be highly coordinated and controlled to elicit appropriate changes in nuclear gene expression and consequent cell replication. This coordination must include important molecular mechanisms that operate to restrain mitogenic signaling as well, since constitutive activation of growth factor pathways would be dysfunctional. Thus, negative feedback mechanisms and internal checks are implicit, if not always explicit, in every important regulatory system involved in cell growth. Furthermore, crosstalk and overlap in receptor intracellular signaling pathways, especially as they apply to heterologous interactions, are powerful means of processing and coordinating external signals to set cell context and generate an appropriate response to the environment. Little is known about the molecular basis of these interactions, however.

Insulin-like growth factor-I (IGF-I) is an essential growth-promoting peptide that shares structural and functional features with insulin (1). We have previously shown that IGF-I is a potent mitogen for normal bovine fibroblasts in culture (2, 3). However, pretreatment with physiologic insulin concentrations rendered these cells refractory to subsequent IGF-I-stimulation of DNA synthesis (3). This insulin-induced desensitization to IGF-I is selective (i.e., no loss of responsiveness to serum), is mediated by specific insulin receptors on bovine fibroblast, and involves events proximal to induction of the nuclear protooncogene, c-myc, and distal to IGF-I receptor binding and activation (3). These data suggested that insulin controlled cell response to IGF-I at an intracellular step along a mitogenic pathway shared by insulin and IGF-I.

The biological effects of insulin and IGF-I are mediated by specific cell surface receptors. These receptors are structurally homologous a,b2 heterotetramers belonging to the family of ligand-activated receptor tyrosine kinases (4–7). Extracellular binding of ligand to receptor a-subunits induces conformational changes and phosphorylation of receptor b-subunits on tyrosines leading to autoactivation of the receptor tyrosine kinase toward specific intracellular proteins. The major receptor substrates include IRS (insulin receptor substrate) and Shc (Src homology 2/collagen) proteins. In most cell systems studied, IRS-1 (~170 kDa) is the predominant substrate phosphorylated in response to stimulation by insulin or IGF-I (8, 9); and several observations suggest a key role for IRS-1 in control of cell proliferation by insulin and IGF-I (10–13). Phosphorylation of IRS-1 on multiple specific tyrosines has the potential for divergent and amplified signaling. Phosphorylated IRS-1 stimulates engagement and activation of phosphatidylinositol 3-ki-

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1 The abbreviations used are: IGF-I, insulin-like growth factor I; IRS, insulin receptor substrate; IL-4, interleukin-4; PI 3-kinase, phosphatidylinositol 3-kinase; MAP kinase, mitogen-activated protein kinase; JAK, Janus kinase; aPY20, anti-phosphotyrosine antibody; SFM, serum-free medium; Shc, Src-homology 2/collagen; PAGE, polyacrylamide gel electrophoresis.

2 C. A. Conover and L. K. Bale, submitted for publication.
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Materials—Recombinant human IGF-I and interleukin-6 (IL-6) were purchased from R & D Systems, Inc. (Minneapolis, MN). Crystalline bovine insulin was kindly provided by Lilly (Indianapolis, IN). IL-4, receptor antibody generated against a synthetic peptide corresponding to tyrosine residues on the IL-4 receptor (23, 25, 28, 29). 4PS has receptor is not a tyrosine kinase itself, but upon activation it tyrosine-phosphorylated substrate of the interleukin-4 (IL-4) receptor (23). Unlike the insulin and IGF-I receptors, the IL-4 receptor is not a tyrosine kinase itself, but upon activation it stimulates an associated Janus kinase, which phosphorylates tyrosine residues on the IL-4 receptor (23, 25, 28, 29). 4PS has also been shown to engage and activate PI 3-kinase (23).

The purpose of this study was to define the molecular events underlying insulin-induced desensitization to IGF-I using the nontransformed bovine fibroblast model. Herein we report a novel role for IRS-2, an intracellular signaling molecule shared by IGF-I, insulin, and IL-4 receptors, in regulating the cells' mitogenic response to IGF-I.

EXPERIMENTAL PROCEDURES

RESULTS

Tyrosine-phosphorylated IGF-I Receptor Substrates—Bovine fibroblasts possess abundant IGF-I receptors and respond to exogenous IGF-I with marked increases in nuclear proto-oncogene expression, DNA synthesis, and cell replication (2, 3). To investigate the initial signaling events in IGF receptor activation, quiescent cultures of bovine fibroblasts were stimulated with 10 nM IGF-I for 10 min and total cell lysates were prepared. Lysate proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with anti-phosphotyrosine antibody (aPY20). With IGF-I treatment, we observed a band at ~97 kDa, and a predominant tyrosyl-phosphorylated species migrating as a broad band between 170 and 190 kDa (Fig. 1). The 170–190-kDa band approximated the size of IRS 1 and we initially presumed it represented IRS-1, the major insulin and IGF-I receptor substrate. However, the majority of tyrosyl-phosphorylated protein in this 170–190-kDa band was immunoprecipitated by antibodies against IRS-1 and not by antibodies against IRS-2 (Fig. 2). The protein immuno-
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Tyrosine phosphorylation in response to IGF-I. Serum-deprived bovine fibroblasts were treated without (−) and with (+) 10 nM IGF-I for 10 min. Total cell lysates were separated by SDS-PAGE and immunoblotted with αPY20. Migration positions of molecular size markers (in kDa) are indicated on the left.

Tyrosine phosphorylation in response to IGF-I: effect of insulin preincubation. Bovine fibroblasts were serum-starved for 48 h without (C) and with 10 nM insulin (ins). Cells were washed and medium changed to SFM containing 10 nM IGF-I, 10 nM insulin, or the combination. [3H]Thymidine incorporation was measured at 22–26 h as described under “Experimental Procedures.” Results are presented as mean ± S.E. of three determinations. *, significant effect of preincubation on IGF-I stimulation, p < 0.05.

Tyrosine phosphorylation in response to IGF-I: effect of insulin preincubation and coinubation. Bovine fibroblasts were serum-starved for 48 h without (C) and with 10 nM insulin (ins), washed, and stimulated without or with 10 nM IGF-I (IGF) for 10 min. Total cell lysates were separated by SDS-PAGE and immunoblotted with αPY20. Migration positions of molecular size markers (in kDa) are indicated on the left.

Tyrosine phosphorylation in response to IGF-I. Serum-deprived bovine fibroblasts were treated without (−) and with (+) 10 nM IGF-I for 10 min. Total cell lysates were separated by SDS-PAGE and immunoblotted with αPY20. Migration positions of molecular size markers (in kDa) are indicated on the left.

Tyrosine phosphorylation in response to IGF-I: effect of insulin preincubation. Bovine fibroblasts were serum-starved for 48 h without (C) and with 10 nM insulin (ins). Cells were washed and medium changed to SFM containing 10 nM IGF-I, 10 nM insulin, or the combination. [3H]Thymidine incorporation was measured at 22–26 h as described under “Experimental Procedures.” Results are presented as mean ± S.E. of three determinations. *, significant effect of preincubation on IGF-I stimulation, p < 0.05.

Insulin-induced Desensitization—As shown in Fig. 4, preincubation but not co-incubation with low concentrations of insulin desensitized bovine fibroblasts to the mitogenic effects of IGF-I. Bovine fibroblasts were washed and changed to serum-free medium for 48 h without or with 10 nM insulin. Cells were washed extensively and then 10 nM IGF-I, 10 nM insulin, or 10 nM IGF-I plus 10 nM insulin added, and [3H]thymidine incorporation determined. IGF-I stimulated [3H]thymidine incorporation 45-fold. Insulin alone produced a much smaller but significant increase in [3H]thymidine incorporation. The co-addition of insulin had no significant effect on IGF-I-stimulated DNA synthesis. However, pretreatment with the same amount of insulin inhibited IGF-I-stimulated [3H]thymidine incorporation by 85%. This insulin-induced cellular desensitization has been characterized previously and cannot be accounted for by changes in IGF-I receptor binding (3).

Investigating where the insulin-induced desensitization occurs along the IGF-I receptor signaling pathway, bovine fibroblasts were treated as in Fig. 4 and total cell lysates immunoblotted for tyrosine-phosphorylated proteins (Fig. 5). IGF-I stimulation was associated with the appearance of bands on αPY20 immunoblots at 97 and 170–190 kDa, as in Fig. 1. Pretreatment with 10 nM insulin for 48 h alone had no visible effect on tyrosine phosphorylation of proteins at 170–190 kDa, but completely prevented the ability of IGF-I to induce phosphorylation of the higher molecular weight bands associated with IRS-2. The lower molecular weight bands in this complex, presumably including IRS-1, did not appear to be affected by insulin pretreatment. A specific decrease in tyrosine-phosphorylated IRS-2 was verified by immunoprecipitation (data not shown). Prolonged insulin treatment also diminished IGF-I-stimulated phosphorylation of the 97-kDa band associated with IGF-I receptor β-subunit, even though some IGF-I receptor-mediated responses are not impaired under these conditions (Ref. 3, and see Fig. 8).

Treatment with 10 nM insulin for 48 h had little or no effect on IRS-1 or IRS-2 protein content as assessed by direct immunoblotting with IRS antibodies (not shown) or by metabolic labeling (Fig. 3). The level of IRS-1 and IRS-2 protein present with and without insulin treatment was nearly equal, as determined by measuring the radioactivity in each band by scintillation counting (Table I). However, following insulin treatment, IRS-2 migrated slower and as a broader band on SDS-
TABLE I

| Preincubation | Incorporated radioactivity (cpm) |
|---------------|----------------------------------|
|               | IRS-1 | IRS-2 |
| Control       | 554   | 857   |
| Insulin       | 808   | 881   |

Fig. 6. IGF-I stimulation of [3H]thymidine incorporation: effect of preincubation with IL-1β, IL-4, and IL-6. Bovine fibroblasts were preincubated for 48 h without (C) and with 10 nm IL-1β (IL-1), IL-4, or IL-6, washed, and then stimulated without or with 10 nm IGF-1, IL-1β, IL-4, or IL-6. [3H]Thymidine incorporation was measured at 22–26 h as described under “Experimental Procedures.” Results are presented as mean ± S.E. of three determinations. *, significant effect of preincubation, p < 0.05.

PAGE. This decrease in mobility, despite the decrease in tyrosine phosphorylation, is often associated with increased serine/threonine protein phosphorylation (10).

Interleukin-4 and IGF-I Receptor Signaling—Since the IL-4 cytokine receptor also has IRS-2 as its major substrate for tyrosine phosphorylation, we assessed the influence of IL-4 in our cell system. As shown in Fig. 6, 10 nm IGF-I stimulated [3H]thymidine incorporation 30-fold, whereas IL-4 showed no significant dose-dependent stimulation. However, pretreatment of bovine fibroblasts with 10 nm IL-4 inhibited IGF-I-stimulated DNA synthesis by 50–60% (p < 0.05) in four separate experiments. Pretreatment with two other cytokines, IL-1β and IL-6, had no effect on IGF-I action in these experiments. In parallel experiments, 10 nm IL-4 was as effective as 10 nm IGF-I in stimulating IRS-2 tyrosyl phosphorylation (Fig. 7A), even though, as noted in Fig. 6, this did not translate into a comparable mitogenic effect. Nonetheless, pretreatment with 10 nm IL-4 inhibited IGF-I-induced IRS-2 phosphorylation. This was not due to down-regulation of IRS-2 since IL-4 had no effect on IRS-2 protein content (Fig. 7B). Pretreatment with IL-1β or IL-6 had no effect on IRS-2 phosphorylation or expression in these cells (data not shown).

Desensitization in the Receptor Signaling Network—IRS-2 has the potential to link IGF-I signaling to both MAP kinase and PI 3-kinase activation. MAP kinase is thought to be the more important pathway for directing IGF-I receptor signaling toward mitogenesis (20); therefore, MAP kinase activity in bovine fibroblasts was measured using a functional in-gel assay. In preliminary time course experiments (not shown), we found that 10 nm insulin and 10 nm IGF-I were equivalent in increasing by 3–5-fold the activity of the p42 and p44 isoforms of MAP kinase. Activation was transient with peak activity occurring within 5 min and returning to near baseline by 90 min. No MAP kinase activity was detectable 24 h after stimulation. This pattern of acute MAP kinase activation is characteristic, and differences in duration of response may influence cell signaling decisions (32, 33). As shown in Fig. 8, IGF-I increased p42/p44 MAP kinase activity 5-fold, and neither the magnitude nor the duration of this stimulation was affected by pretreatment with insulin for 48 h. Similarly, we saw no effect of IL-4 pretreatment on the ability of IGF-I to activate the MAP kinase pathway (data not shown). Therefore, despite activation of the MAP kinase signaling pathway, IGF-I-stimulated mitogenesis did not occur if preceded by insulin or IL-4 receptor activation. That insulin-induced cellular desensitization does not involve the MAP kinase signaling pathway was supported by experiments using PD98059, a specific inhibitor of MAP kinase activation by upstream MAP kinase kinase (34). Although we could show that PD98059 was an effective inhibitor of insulin-stimulated MAP kinase activity in our system, the presence of PD98059 during insulin pretreatment did not prevent cellular desensitization to subsequent IGF-I stimulation (Table II). PD98059 did not have an independent inhibitory effect since pretreatment for 48 h with PD98059 alone did not interfere with IGF-I stimulation.

On the other hand, insulin-induced desensitization to IGF-I could be prevented if LY294002, a specific inhibitor of PI 3-kinase activation (35), was present during the preincubation period (Fig. 9). In three separate experiments, IGF-I stimulation following preincubation with insulin and LY294002 was 94–106% of maximum versus 21–25% of maximum following preincubation with insulin alone. LY294002 pretreatment in the absence of insulin produced a decrease in IGF-I-stimulated [3H]thymidine incorporation, which appeared to reflect a residual effect of the inhibitor on IGF-I receptor signaling (data not shown). Retention of IRS-2 tyrosine phosphorylation was concomitant with the IGF-I-stimulated DNA synthesis following preincubation with insulin and LY294002 (data not shown). Interestingly, LY294002 also prevented the decrease in mobility and broadening of the IRS-2 band on SDS gels in response to prolonged insulin treatment (Fig. 10).
Cultures were washed and IGF-I-stimulated [3H]thymidine incorporation measured as described in the legend to Fig. 4. Results are mean ± S.E. of three determinations.

| Preincubation | % [3H]thymidine incorporation | Control | IGF-I |
|---------------|--------------------------------|---------|-------|
| Control       | 0.19 ± 0.017                  | 7.92 ± 0.244 |
| Insulin       | 0.20 ± 0.011                  | 1.34 ± 0.092 |
| PD98059       | 0.12 ± 0.012                  | 7.32 ± 0.458 |
| Insulin + PD98059 | 0.09 ± 0.004        | 1.32 ± 0.108* |

* Significant effect of preincubation on IGF-I stimulation, p < 0.05.

**DISCUSSION**

The control of cell growth is complex, involving coordinated integration of signals arising from a variety of activated receptors. The vast majority of studies use transfected or immortalized cells overexpressing a particular receptor to delineate its signal transduction pathway for mitogenesis. This approach has been crucial to our present understanding of cell growth. Nevertheless, there is a growing appreciation that feedback loops and intracellular cross-talk between signaling pathways may underlie natural mechanisms of receptor activity regulation, and that “normal” cell models may be more relevant to these types of investigations. Cultured bovine fibroblasts, naturally expressing a high number of IGF-I receptors, have been a useful cell model for studying various aspects of IGF physiology (2, 3, 36, 37). In the present study we identified signaling cascades involved in IGF-I-induced mitogenesis in these cells, characterized the interplay among IGF-I, insulin, and cytokine receptor signaling, and produced evidence of a distinct role for IRS-2 in cell growth regulation.

**IGF-I, Insulin, and Cytokine Receptor Signaling in Normal Bovine Fibroblasts**—Cultured bovine fibroblasts possess classic IGF-I tyrosine kinase receptors with mitogenic end points similar to those reported for insulin tyrosine kinase receptors, proto-oncogene expression, DNA synthesis, and cell replication (2, 3). We found the initial signaling events to be similar as well, i.e. autophosphorylation of the receptor β-subunit on tyrosines and subsequent tyrosine phosphorylation of specific intracellular substrates, primarily IRS. Interestingly, the major IRS protein tyrosine-phosphorylated in response to IGF-I in bovine fibroblasts was IRS-2, rather than IRS-1. IRS-1 is expressed by these cells, however, as determined by metabolic labeling. No other IGF-responsive tyrosine-phosphorylated proteins were evident under our conditions. Phosphorylation of Shc protein represents an IGF-I-stimulated signaling event alternative to IRS (8, 9, 20). However, there was no specific increase in tyrosine-phosphorylated proteins of 46, 52, and 66 kDa that could represent Shc proteins and no specific protein immunoprecipitated with αShc antibodies (data not shown), suggesting low abundance of Shc in these cells.

Insulin receptors also mediate proto-oncogene expression and DNA synthesis in bovine fibroblasts. However, pre-exposure to low concentrations of insulin that do not interfere with IGF-I receptor binding renders the cells refractory to subsequent IGF-I-stimulated DNA synthesis (3). Under these conditions, preincubation with insulin completely blocked the IGF-I-induced increase in tyrosine-phosphorylated 190-kDa IRS-2. The tyrosyl-phosphorylated band at 170 kDa, presumably IRS-1, was relatively unaffected by insulin. These data implicate IRS-2 in insulin/IGF-I post-receptor interplay, and suggest that IRS-2, and not IRS-1, is involved in insulin-induced desensitization to IGF-I in this model. Giorgino and Smith (38) similarly concluded that IRS-1 is not involved in dexamethasone-induced potentiation of IGF-I receptor signaling in muscle cells.

IL-4, a pluripotent cytokine, was equivalent to IGF-I in rapid tyrosine phosphorylation of IRS-2 in bovine fibroblasts. This effect was not seen with the other interleukins tested (IL-1β and IL-6). Unlike IGF-I and insulin receptors, the IL-4 receptor does not possess intrinsic tyrosine kinase activity and presumably gains this function through association with Janus kinases (23, 25, 28, 29). IL-4-induced IRS-2 phosphorylation was not associated with a mitogenic response in these cells. Nonetheless, IL-4 pretreatment inhibited both IGF-I-stimulated DNA synthesis and IGF-I-induced IRS-2 tyrosine phosphorylation, suggesting that IRS-2 is not sufficient for mitogenic signaling in this system but that it may be necessary for mitogenic signaling by other growth factors. Alternatively, IGF-I and IL-4 tyrosine phosphorylation of IRS-2 may be qualitatively different (39). Furthermore, these data indicate that preactivation of a mitogenic signaling pathway is not essential for desensitization, since IL-4 was able to induce cellular resistance without affecting DNA synthesis. Although the IL-4 receptor is structurally and functionally distinct from the insulin and IGF-I receptors, all three share a common motif in their juxtamembrane region that may directly or indirectly interact with IRS protein (40). It will be important to determine whether this domain is essential for heterologous receptor desensitization. There are other recent examples where cytokines have been shown to influence cell growth responses in vitro and in vivo. Leptin, an adipocyte-derived cytokine, attenuated insulin-induced tyrosine phosphorylation of IRS-1 and stimulation of gluconeogenesis in HepG2 cells (41). Tumor necrosis factor α is an important mediator of insulin resistance in muscle and fat tissue (42). Collectively, these data indicate significant interplay among IGF-I, insulin, and cytokine receptor signaling, which may represent part of normal physiologic cell growth regulation.

**FIG. 9. Effect of LY294002 on insulin-induced desensitization to IGF-I**. Bovine fibroblasts were preincubated for 48 h without (C) or with 10 nM insulin (ins), 50 μM LY294002 (LY), or the combination. Cultures were washed and IGF-I-stimulated [3H]thymidine incorporation measured. Results are mean ± S.E. of three determinations.

**FIG. 10. Metabolic labeling: effect of LY294002.** Cells were pre-treated with 10 nM insulin (lanes a-d) as in Fig. 3, except that LY294002 (50 μM) was included in the insulin preincubation period (lanes b and d). Total cell lysates were immunoprecipitated with αIRS-2 or nonspecific IgG, and run on a 5–15% SDS-polyacrylamide gel. 32S-Labeled proteins were detected by autoradiography.
**IRS-2 as a Functional Locus for Cellular Desensitization**—Our data suggest that IGF-I, insulin, and IL-4 receptor signaling pathways converge at a step where regulation of IRS-2 determines cellular sensitivity. Our findings of unaltered IGF-I-stimulated amino acid transport and glucose uptake (3) and of IGF-I-stimulated MAP kinase activation following prolonged pre-exposure to low concentrations of insulin or IL-4, suggest a functional IGF-I receptor and primary check point beyond receptor autophosphorylation. However, decreased tyrosine phosphorylation of the 97-kDa β-subunit of the IGF-I receptor may indicate possible upstream effects of receptor autophosphorylation that will need to be addressed. Furthermore, unlike models of chronic stimulation with 10–100-fold higher concentrations of insulin (19, 42–44), down-regulation of IRS-2 (or IRS-1) expression did not occur. In our system, desensitization is also unlikely to be the result of simple competition for a limiting substrate because co-treatment as opposed to pretreatment with insulin or IL-4 had no effect on IGF-I stimulation of DNA synthesis or IRS-2 phosphorylation on tyrosines, although both insulin and IL-4 alone stimulate IRS-2 tyrosine phosphorylation. Thus, time appears to be an important dimension of signal transduction by virtue of its impact on intracellular response patterns which may permit the defined order of the cell cycle to proceed.

The decrease in tyrosine phosphorylation of IRS-2 appears to be central to cellular desensitization to IGF-I. This could be accomplished by dephosphorylation of phosphorytrosines on IRS-2 or, alternatively, by phosphorylation on serine/threonines. Phosphorylation of signal elements by activated downstream serine/threonine kinases has been shown to inhibit subsequent tyrosine phosphorylation of the substrate (18, 45). What was striking in our metabolic labeling studies was that insulin-induced cellular desensitization was invariably accompanied by an increase in IRS-2 apparent molecular mass on SDS gels. Sun et al. (10) demonstrated that a similar molecular mass shift in IRS-1 after prolonged insulin treatment of Chinese hamster ovary cells transfected with human IRS-1 reflected an increase in degree of serine phosphorylation of the protein. Phosphorylation of IRS-1 on serine and threonine residues interfered with the subsequent tyrosine phosphorylation of IRS-1 by insulin receptors in this system. Our model may be analogous to that activation of insulin receptor or IL-4 receptor signaling may cause increased expression or activation of a downstream kinase which phosphorylates IRS-2, thereby preventing subsequent tyrosine phosphorylation by IGF-1 receptors. LY294002, an inhibitor of dual specificity PI 3-kinase that possesses both lipid and serine kinase activities (17). However, our preliminary experiments (not shown) failed to reveal any change in protein-tyrosine phosphate 1B with insulin treatment of bovine fibroblasts as assessed by immunoblot.

As noted above, inhibition of PI 3-kinase activation by LY294002 blocked the ability of insulin to induce desensitization to IGF-I, implicating the PI 3-kinase signal pathway in cellular desensitization to IGF-1-stimulated mitogenesis. Although MAP kinase is considered a key mitogenic signaling pathway (9, 20, 21), MAP kinase activation was not sufficient to propagate IGF-I-stimulated mitosis in bovine fibroblasts. Furthermore, given the lack of effect of insulin pretreatment on IGF-I-stimulated MAP kinase activity, it seems unlikely that desensitization occurs within the cascade leading to activation of MAP kinase. The latter was supported by the finding that a potent inhibitor of MAP kinase activation, PD98059, did not prevent desensitization during pretreatment with insulin.

**Conclusions**—The impaired response of bovine fibroblasts to IGF-I as a consequence of pretreatment of cells with insulin or IL-4 is associated with post-receptor signaling alterations at the level of IRS-2. The molecular mechanism for the decrease in IGF-2 tyrosine phosphorylation in response to IGF-1 under these conditions is not clear, but the data suggest a feedback mechanism mediated by a preactivated receptor tyrosine kinase pathway (i.e. by insulin or IL-4) that induces serine phosphorylation of IRS-2 which, in turn, interferes with IGF-I receptor-stimulated signal transduction through IRS-2 in these cells. IRS-2 is expressed in many cell types, and is not peculiar to bovine fibroblasts, suggesting a role in normal insulin, IGF-I, and IL-4 signaling (39, 47). Bovine fibroblasts appear to be a particularly useful model for further studies in this regard. Better understanding IRS-2 will be of critical importance to our understanding of integrated cell signaling and post-receptor desensitization, and perhaps be of relevance to growth-resistant states produced by insulin and cytokine receptor activation.

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