

Early insulin signaling cascade in a model of oxidative skeletal muscle: mouse Sol8 cell line

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Abstract

Cell models provide important tools to investigate the mechanisms modulating the insulin-signaling cascade. Insulin interaction and subsequent signaling of cells is complex and regulated at multiple levels: receptor abundance, binding dynamics, phosphorylation/dephosphorylation of tyrosine and serine/threonine residues, and subsequent interactions of key intracellular messengers. We report early insulin signaling events in the mouse Sol8 myogenic cell line. Sol8 cells responded to insulin by increasing total IRS-1, p85 PI3-kinase and tyrosine phosphorylated IRS-1 (pY-IRS-1) at 10 min ($P < 0.05$), but not at 1 min of insulin stimulation. The dose–response relationships at 10-min insulin (10 to 300 nM) stimulation showed that IRS-1 and pY-IRS-1 responded to 100 and 300 nM insulin, and the p85 PI3-kinase response peaked at 30 nM insulin. PI3-kinase appeared to be present in high abundance and, in response to insulin, recruitment to the insulin receptor tyrosine kinase (IR) of IRS-1 and PI3-kinase was observed. The increase in IRS-1 detected in IR immunoprecipitates was twofold, while the corresponding increase in PI3-kinase was threefold, suggesting direct recruitment of PI3-kinase to the IR. PI3-kinase detected in IRS-1 immunoprecipitates in response to insulin increased 1.7-fold. An ultimate target of this pathway, GLUT4 recruitment to the PM, was delayed (30 min), the increase in GLUT4 being of similar magnitude (1.6-fold) to the early signaling events. Saturation binding analysis indicated that IR in the plasma membrane was not down-regulated in response to insulin. The present study suggests that early signaling events in the insulin cascade are invoked in Sol8 myogenic cells and that this cell line provides a useful model to study insulin signaling. © 2004 Elsevier B.V. All rights reserved.

Keywords: Signal transduction; Insulin; Insulin receptor; IR; IRS-1; PI3-kinase; Sol8; Muscle

1. Introduction

Skeletal muscle is the major metabolic tissue of the body. Muscle functions to allow limb movement, but it also provides a readily available source of metabolites for use by other tissues such as bone and brain. As insulin operates to facilitate normal muscle metabolism, muscle is an important tissue in the study of insulin signaling, for which there are many models available. For example,

cultured myogenic cells isolated from human subjects with high or low insulin sensitivity [1], immortalized cell lines such as the mouse C2C12 [2], BC3H-1 [3], or the rat L6 [4, 5], have been used as skeletal muscle models, each with its own strengths and weaknesses. The Sol8 myogenic cell line is derived from oxidative (soleus) muscle [6]. Sol8 cells are characterized by having large numbers of mitochondria, which allows these cells to readily oxidize fatty acids as an energy source although the pathways of action are not yet well described [7]. Modulation of energy flux in Sol8 cells is regulated, in part, by insulin, and the potential to utilize fatty acids rather than glucose for energy suggests that Sol8 may exhibit insulin resistance. To date, only limited aspects of

Abbreviations: IR, insulin receptor kinase; IRS-1, insulin receptor substrate-1; PI, phosphatidylinositol

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the insulin-signaling cascade have been described in this cell model [8].

In the insulin-signaling cascade, the potential for variation in downstream signal recruitment via IRS is substantial. IRS proteins associate with the IR NPEY motif via a phosphotyrosine-binding domain (PTB) together with ancillary membrane phosphoinositide binding via the IRS pleckstrin homology (PH) domain. IRS-1 also possesses at least eight C-terminal tyrosine phosphorylation sites, together with a potential counter-regulatory serine-307 phosphorylation site adjacent to the PTB domain [9–14]. Direct binding of PI3-kinase to the IR has been demonstrated in both muscle and liver [15–22] and this mechanism has been proposed to have counter-regulatory effects on insulin signaling in gestational diabetes [20]. The objective of the present study was to characterize early insulin signaling events in Sol8 cells, and explore possible inhibition of signaling.

2. Methods

2.1. Myogenic cells and cell culture

Sol8 myogenic cells were cultured in DMEM with 10% FCS plus penicillin/streptomycin (CM) in a humidified environment at 37 °C and 5% CO₂ enriched air. Cultures were expanded and allowed to grow to 80–90% confluence in an undifferentiated state. For insulin treatments, cultures were grown to subconfluence in CM, washed in DMEM (3×) and the insulin treatments applied in DMEM plus 0.3 mM BSA, fatty acid-free (Sigma Chemical, St Louis, MO).

2.2. Preparation of cell lysates

Following experimental treatments, cultures were washed (3×) in DMEM, and 15 ml of lysis buffer, 50 mM Tris, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, plus ‘Complete mini-cocktail (Roche Diagnostics, GmBH, Germany) pH 7.3, was added. Culture flasks were then immersed in liquid nitrogen for 30 s to ensure complete cell lysis. Cell lysates were then incubated at 4 °C for 2 h, then aliquoted and stored at –80 °C. Total protein was determined using the BCA assay (Pierce Chemical, Rockford, IL) and DNA was extracted from an aliquot to quantify cell number.

2.3. Immunoprecipitation

For each specific insulin cascade protein studied, 5 µl of specific antibody was preincubated with 20-µl Protein-A Sepharose (Pierce) for 2 h at 4 °C, and 1 ml of cell lysate was then added and incubated with constant agitation overnight at 4 °C. The mixture was then centrifuged at

14 000×g for 2 min, the supernatant aspirated and the pellet resuspended in 1-ml lysis buffer. The beads were washed a further two times and finally suspended in 50-µl non-reducing Laemmli buffer. The specific immunoprecipitating antibodies (Upstate Biotechnology, Lake Placid, NY) were each raised in rabbits and were directed against either the IR β-chain, IRS-1 or PI3-kinase (p85).

2.4. Electrophoresis and immunodetection

Proteins in immunoprecipitates were resolved using SDS-PAGE under non-reducing conditions (Hoefer Scientific Instruments, San Francisco, CA), transferred to nitrocellulose (Semi-dry Transfer, Hoefer, Model TE-70), and the proteins detected using a rabbit antibody as shown for immunoprecipitation as primary antibody (1:1000), and mouse monoclonal (clone 4G10) anti-phosphotyrosine (Upstate Biotechnology) (1:1000) to detect tyrosine phosphorylated proteins. Blots were incubated simultaneously with dual secondary antibodies (1:5000): Alexa Fluor 680-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and IRDye 800-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA). Total specific proteins and phosphorylated proteins were simultaneously detected using an ‘Odyssey’ dual infrared laser fluorescence imager (Li-Cor Biosciences, Lincoln, NE) with resolution set to 169 µm.

2.5. Plasma membrane preparation and GLUT4 analysis

Following insulin treatments, plasma membranes were prepared through a sucrose cushion as described [23], and 100-µl aliquots stored at –80 °C. Protein content was determined using the BCA assay with bovine serum albumin as the standard (Pierce). GLUT4 was immunoprecipitated using an anti-GLUT4 antibody (Abcam, Cambridge, UK), electrophoresed, blotted under reducing conditions and detected as described above.

2.6. Saturation binding analysis

Insulin binding to IR was quantified on whole cells using saturation binding analysis. Bovine insulin was iodinated by the method of Salacinski et al. [24]. Cells were grown to 80–90% confluence, and not treated or treated with insulin (100 nM). Cells were then washed (3×) with DMEM and incubated for 2 h at room temperature in the presence of six concentrations (in triplicate) of ¹²⁵I-insulin alone (concentration range 30 to 2000 pM) to determine total binding, or with the addition of 500 nM insulin to define nonspecific binding, in 250 µl of a buffer containing 100 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1 mM EDTA, 10 mM D-glucose, 15 mM sodium acetate, 0.1% BSA, plus protease inhibitor cocktail (Roche Diagnostics) pH 7.6 [25, 26]. Bound and free ligands were separated by centrifugation and resuspension of cells (2×) at 3000×g for 20 min. Bound

radioactivity was quantified using a gamma counter (Packard, Meridan, CT). Data were modeled using the Ligand Binding Module in SigmaPlot version 8 (SPSS, Chicago, IL), and IR affinity and receptor number determined.

2.7. Experimental treatments and design

In Experiment 1, a time-course response to insulin was determined. Cell cultures were prepared in 150-cm² flasks and incubated in the presence of bovine insulin (Gibco InVitrogen, Carlsbad, CA), 100 nM for either 0, 1, 10 or 30 min. Cells were also maintained in CM and processed together with insulin-treated cells as outlined above. In Experiment 2, dose–response to insulin was determined. Cells were incubated for 10 min with the following concentrations of insulin (nM): 0, 10, 30, 100, and 300, or in CM, and pathway activation determined as for experiment 1. For each experiment, control vs. treatments were resolved and blotted from the same gel and each of these was replicated three to six times. Only within blot data are reported (paired *t* test).

3. Results

3.1. Insulin response of IRS-1 and p85 PI3-kinase

In both experiments 1 (time course) and 2 (dose–response), total IRS-1 and PI3-kinase were responsive to

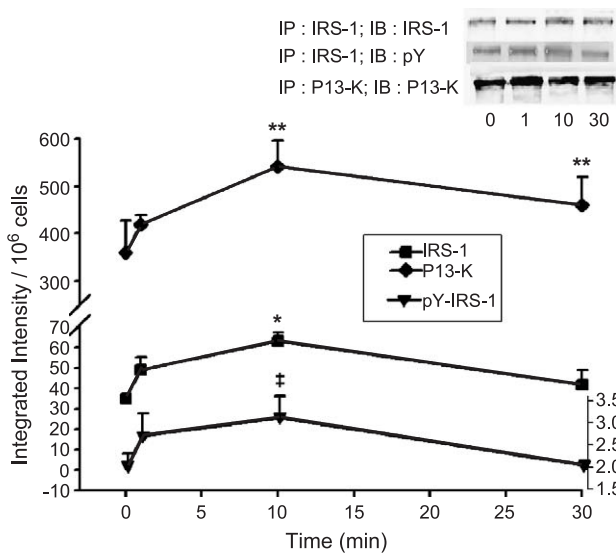


Fig. 1. Effect of insulin (100 nM) on total IRS-1 (■, left axis), tyrosine phosphorylated IRS-1 (pY-IRS-1; ▼, right axis) and p85 PI3-kinase (◆, left axis) in Sol8. (Note different scales.) Cells were stimulated with insulin for the indicated times, lysed and IRS-1 or p85 PI3-kinase immunoprecipitated using an excess of specific antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, Western-blotted using the same antibody for total specific proteins or anti-phosphotyrosine for pY-IRS-1, and proteins quantified using the Odyssey dual infrared laser fluorescence system ([‡]*P*<0.1, **P*<0.05, ***P*<0.01, vs. 0 insulin, *n*=3). Representative blots from each study are shown.

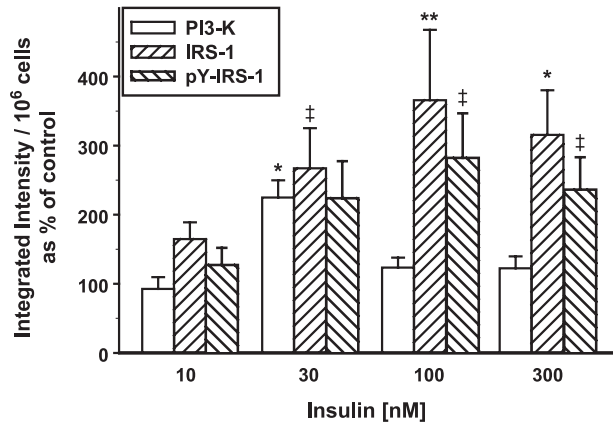


Fig. 2. Dose–response effects of insulin on IRS-1 and p85 PI3-kinase in Sol8 cultures. Cells were stimulated with insulin for 10 min at the indicated concentrations, lysed and IRS-1 or PI3-kinase immunoprecipitated using an excess of specific antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, Western-blotted using the same antibody for total specific proteins or anti-phosphotyrosine for pY-IRS-1, and proteins quantified using the Odyssey dual infrared laser fluorescence system. Data were expressed as percent of the control value for each protein form ([‡]*P*<0.1, **P*<0.05, ***P*<0.01, vs. 0 insulin, *n*=5–6).

insulin (Figs. 1 and 2). In the time-course study, the rise in both IRS-1 and PI3-kinase peaked at 10 min (*P*<0.05; <0.01, respectively), PI3-kinase remaining elevated (*P*<0.01), at 30 min of insulin stimulation. In experiment 2, maximal IRS-1 response was detected at 100 and 300 nM insulin, while PI3-kinase appeared responsive only to 30 nM insulin. Total IRS-1 was closely reflected by pY-IRS-1 in both studies also showing a peak at 10 min in the time-course study (*P*<0.1) and in experiment 2 in response to 100 and 300 nM insulin (*P*<0.1). Although abundance of total IRS-1 and PI3-kinase is not a direct measure of insulin signaling, the increase in total messenger indicates an insulin-specific action on either specific protein synthesis or inhibition of degradation. A direct indicator of insulin action, tyrosine phosphorylated IRS-1, appeared to be only weakly responsive to insulin.

3.2. Recruitment of IRS-1 and p85 PI3-kinase to IR

Consistent with the temporal response of total pY-IRS-1, IRS-1 recruited specifically to IR peaked at 10-min insulin stimulation (Fig. 3, *P*<0.05). The temporal response of PI3-kinase recruited to IR also peaked after 10-min insulin (*P*<0.05).

3.3. Recruitment of p85 PI3-kinase to IRS-1

In Sol8, recruitment of PI3-kinase to IRS-1 showed a consistent temporal pattern with the observations reported above. The response appeared to peak after 10 min of insulin stimulation (*P*<0.01, Fig. 4). Thus, insulin signaling from IR through IRS-1 and PI3-kinase, the classical pathway of early insulin cascade signaling, may

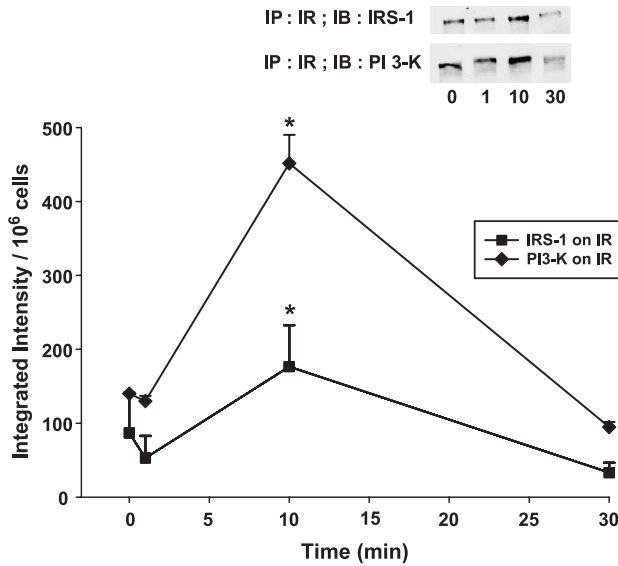


Fig. 3. Effect of insulin (100 nM) on recruitment of IRS-1 (■) and p85 PI3-kinase (◆) to the insulin receptor (IR) in Sol8 cultures. Cells were stimulated with insulin for the indicated times, lysed and proteins immunoprecipitated using an excess of anti-IR antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, co-precipitated proteins Western-blotted using either anti-IRS-1 or anti-p85 PI3-kinase and quantified using the Odyssey dual infrared laser fluorescence system (* $P < 0.05$ vs. 0 insulin, $n = 3$). Representative blots from each study are shown.

have reached a peak at 10-min insulin, was down-regulated and apparently recalcitrant to insulin at 30-min incubation.

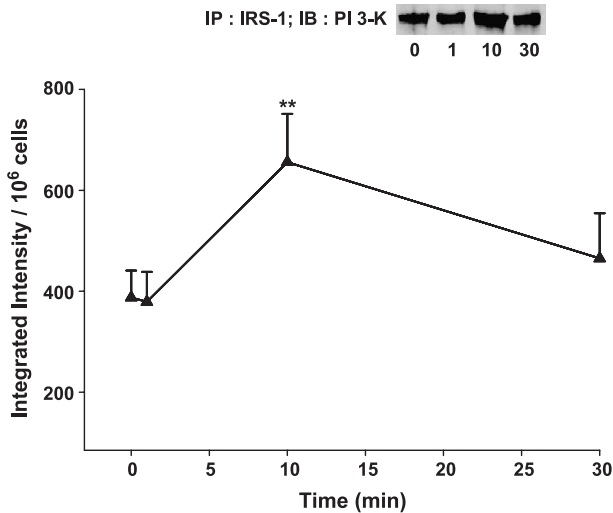


Fig. 4. Effect of insulin (100 nM) on p85 PI3-kinase recruitment to IRS-1. Cells were stimulated with insulin for the indicated times, lysed and proteins immunoprecipitated using an excess of anti-IRS-1 antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, and co-precipitated p85 PI3-kinase was Western-blotted and quantified using the Odyssey dual infrared laser fluorescence system (** $P < 0.01$ vs. 0 insulin, $n = 6$). A representative blot is shown.

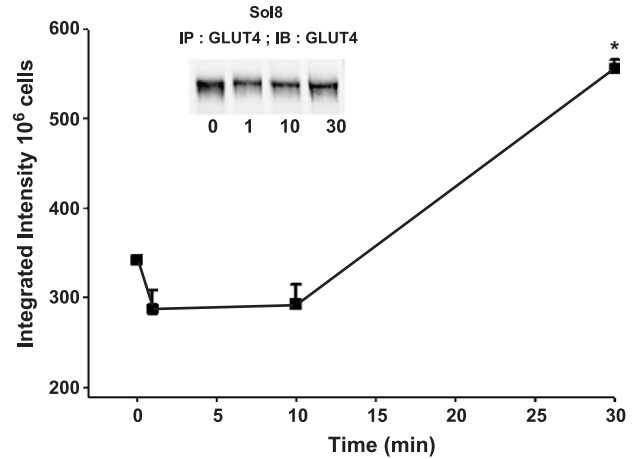


Fig. 5. Effect of insulin (100 nM) on recruitment of GLUT4 to the plasma membrane in Sol8. Cells were stimulated with insulin for the indicated times and lysed. Plasma membranes were prepared and proteins immunoprecipitated using an excess of anti-GLUT4 antibody bound to Protein-A agarose. Immunoprecipitates were electrophoresed, Western blotted using the same antibody and proteins quantified using the Odyssey dual infrared laser fluorescence system. (* $P < 0.05$ vs. 0 insulin, $n = 3$). A representative blot is shown.

3.4. GLUT4 recruitment to the plasma membrane

To examine the effects of insulin on an important target of the IR–PI3-kinase pathway, plasma membranes were prepared, and the levels of GLUT4 quantified. Early variation in plasma membrane GLUT4 was not detected, but by 30 min following insulin stimulation, GLUT4 was elevated more than 1.6-fold ($P < 0.05$, Fig. 5). This observation is also consistent with the delayed response expected, following early signaling events at around 10 min post-insulin stimulation. These data appear to be in agreement with the insulin-sensitive state of Sol8, with GLUT4 recruitment increasing over time, although earlier signaling events had peaked and returned to basal values.

3.5. Saturation binding analysis of IR

The effect of 10-min insulin (100 nM) stimulation on IR number (B_{\max}) and affinity (K_D) in Sol8 was examined. The K_D fell within the expected range and was not affected by insulin treatment (control, 2.6 ± 2.3 nM; insulin treated, 4.8 ± 2.5 nM). In addition, B_{\max} was apparently unchanged by insulin treatment (control, 10.9 ± 7.3 pM; insulin treated, 20.0 ± 17.0 pM).

4. Discussion

Sol8 myogenic cells provide a useful model for the study of the complex pathways activated by insulin. Insulin acts on multiple cellular processes including the regulation of energy uptake and partitioning, amino acid uptake and progression to mitosis and differentiation. Although one

aspect of insulin signaling, activation of differentiation, was recently reported in the Sol8 model [8], there have been no reports of early insulin signaling in this model.

Recruitment and activation of IRS-1 and p85 PI3-kinase occurs in response to insulin although each of these messengers may be involved in multiple pathways [27]. Thus, these messengers are part of the complex interplay between signals arising at the cell membrane and of the regulation of cellular metabolism. Sol8 cells, being derived from oxidative muscle, have the potential to readily utilize fatty acids or glucose as energy substrate and thus provide a model in which switching between the hormone pathways regulating energy substrate partitioning has the potential to invoke insulin resistance [28].

Sol8 early insulin signaling appeared to be responsive, showing tyrosine phosphorylation of IRS-1, recruitment of IRS-1 to the IR, and p85 PI3-kinase recruitment to IRS-1. The order of magnitude of each of these events in response to insulin was similar, increasing 1.5- to 2-fold over basal levels. However, it appears that PI3-kinase may be present in great abundance in these cells (Fig. 1), and direct recruitment of PI3-kinase to the IR may be a mechanism involved in the fine-tuning of the insulin response.

Recent *in vivo* or *in vitro* studies of early events in the insulin signaling cascade report maximal response times as 2.5 min [29] to 5 min [20,30,31]. Although none of these studies reported an earlier time-point, an earlier study reported rapid phosphorylation of the IR and IRS-1 (30 s) in rat liver, and a slow decrease in IR phosphorylation, with $t_{1/2}=6$ min [32]. A study of a similar rat liver model found variations in levels of IRS-1 and associated PI3-kinase in plasma membrane, endosomes and cytosol within 30 s and 2 min of insulin stimulation [33], which returned to basal levels by 15 min post-insulin. In the present study, it appears that the cells were unresponsive to 1-min insulin stimulation, which may reflect in part a difference between the *in vitro* model reported in the present study and these *in vivo* studies. The lack of responsiveness of Sol8 at the 30 min time-point is consistent with the above studies, indicating that the signaling pathway is down-regulated, and indicating a normal response to high insulin concentrations rather than insulin resistance. It should be noted that at the 10-min time-point, peak response to insulin may have passed. The precise peak of response appears to vary between models, but appears to be invoked from as rapidly as 30 s [32] and be maintained for up to 15 min [33]. Thus, it is possible that the kinetics of the insulin response in the present study is similar to those reported in *in vivo* studies cited above. However, at 1 min of insulin stimulation, no response in early insulin signaling was detected.

A mechanism believed to down-regulate insulin signaling—the direct binding of p85 PI3-kinase to the IR [20–22]—may be invoked in Sol8. Co-precipitation of both IRS-1 and p85 with the IR may occur via a number of possible mechanisms. p85 may be bound to pY-IRS-1 associated with pY-IR and direct binding of p85 to pY-IR is possible.

The high affinity binding site, pYMxM, recognized by the SH2 domains of p85, occurs at five sites on murine IRS-1 [34]. Furthermore, the activated mouse IR (and the type 1 IGF receptor) do not possess such a high affinity site but have a lower affinity, C-terminal binding site, pYTHM, which is also recognized by p85 SH2 domains. It appears that the high abundance of p85 PI3-kinase in Sol8, even in those not stimulated with insulin, may be a factor in modulation of insulin signaling. Although it is difficult to precisely quantify the insulin cascade proteins using the methods utilized here, it does appear that an excess abundance of PI3-kinase over IRS-1 may contribute to the mechanism of fine-tuning the intracellular response to insulin. The relative affinity of pYMxM on IRS-1 versus that of the pYTHM site on the IR may affect the distribution of PI3-kinase between the IR and IRS-1. The distribution would also be affected by the relative concentrations of each of these messengers adjacent to the plasma membrane. Differences in these affinities and basal concentrations of each messenger may explain subtle differences in recruitment patterns in similar sub-plasma membrane compartments. At the peak time-point (10 min) in response to insulin, PI3-kinase recruitment to the IR represented a threefold increase from basal, which had returned to basal level by 30-min insulin stimulation. The corresponding increase in IRS-1 recruitment to the IR represented a twofold increase. It appears that although these cells are responsive to insulin, direct recruitment of PI3-kinase to the IR may provide a mechanism to modulate signaling. Quenching of the response to this hyperphysiological concentration of insulin was clearly invoked by 30-min insulin stimulation.

Recruitment of IRS-1 in the present study shows some subtle differences from other models. In an *in vivo* study in mouse gastrocnemius muscle, Shao et al. [20] demonstrated an approximately fivefold increase in pY-IRS-1 in normal versus a threefold increase in gestational diabetes. In comparison, pY-IRS-1 peaked at approximately 1.5-fold basal in the present study on a background of increasing total IRS-1. In contrast, there was apparently no change in total IRS-1 in either group in the previous study [20] in response to insulin. Further investigation is required to clarify the importance of these apparent differences.

GLUT4 recruitment to the plasma membrane (PM) is delayed following early insulin-signaling cascade events, the response observed in the present study being consistent with other insulin-sensitive models. An *in vivo* study in normal and GLUT4 transgenic mice [35] showed in normal mice, in response to a maximal dose of insulin, that GLUT4 recruitment to the PM increased by approximately 2.5-fold. In a later report [36], these workers noted the possibility that an activation process may be required to describe functional glucose transporters accurately; a view supported by others [37]. Other *in vivo* studies of GLUT4 response in insulin-sensitive models indicate that a two- to fivefold increase in PM GLUT4 in response to insulin may be expected [38–41].

Thus, it appears that the GLUT 4 recruitment response in Sol8 is consistent with normal insulin sensitivity.

A number of studies have reported that down-regulation of IR following insulin stimulation requires several hours [42–44]. Thus, little change in receptor number would be expected during the 1- to 30-min insulin stimulation period used in the present study. Furthermore, the K_D values are in close agreement with others [43]. We have not found other estimates of receptor number in myogenic cells in the literature. The values reported here (range 11 to 20 pM, equivalent to 3 to 6 fmol per 10^6 cells), is somewhat in excess of values reported for small, chromaffin cells, 0.25 fmol per 10^6 cells [43,45].

In conclusion, early insulin-cascade signaling in Sol8 myogenic cells is responsive to insulin. These cells, being derived from an oxidative muscle (soleus), provide a useful model for exploring partitioning of energy substrate utilization processes, through which early insulin signaling events interact with other regulatory axes.

Acknowledgments

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