Bi-directional Regulation of Brown Fat Adipogenesis by the Insulin Receptor

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Insulin is a potent inducer of adipogenesis, and differentiation of adipocytes requires many components of the insulin signaling pathway, including the insulin receptor substrate IRS-1 and phosphatidylinositol 3-kinase (PI3K). Brown pre-adipocytes in culture exhibit low levels of insulin receptor (IR), and during differentiation there is both an increase in total IR levels and a shift in the alternatively spliced forms of IR from the A isoform (−exon 11) to the B isoform (+exon 11). Brown adipocytes contain a unique isoform of IR that contains exon 11 in both the A and B isoforms. Although white adipose tissue is the primary site for lipid storage and fatty acid release, brown adipocytes are uniquely responsible for basal thermogenic energy expenditure through expression of the mitochondrial protein uncoupling protein-1 (UCP-1). Recently, significant progress has been made in clarifying the molecular mechanisms of adipocyte differentiation, which includes the expression and activation of a series of transcription factors, including the C/EBP family members β, δ, and α and the peroxisome proliferator-activated receptor gamma (PPARγ). Expression and activation of these transcription factors are required for the expression of adipogenic markers such as fatty acid synthase (FAS) and the insulin-sensitive glucose transporter Glut4.

Upstream signals regulating the expression and activation of these transcription factors during adipocyte differentiation are not fully understood. However, the derivation of white and brown adipocyte cell lines that are deficient in insulin or insulin receptor signal transduction suggests that these transcription factors are key to adipogenesis. Here we examine the role of IR in adipogenesis using two mouse lines that are deficient in IR. We find that brown adipocytes that overexpress IR. We find that brown adipocytes

This article has been withdrawn by the authors. The authors were recently made aware of duplicated images of PCR reaction products in panels B and D in Fig. 2. These duplications were inadvertently introduced during figure assembly. Review of the original data generated in the lab at that time (2000–2002), as well as subsequent studies, confirmed the conclusions of the manuscript. However, in the interest of maintaining accuracy in the published scientific literature and because the initial figures were not up to the standards of JBC, the authors wish to withdraw this article. The authors apologize for these errors.
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EXPERIMENTAL PROCEDURES

Materials—Antibodies used for immunoprecipitation and immunoblotting included anti-IRS-1, anti-IRS-2, and anti-phosphotyrosine 4G10 (kindly provided by Morris White, Joslin Diabetes Center, Boston, MA); anti-insulin receptor (kindly provided by Bentley Cheatham, Joslin Diabetes Center); anti-PPARγ, anti-iC/EBPα, and anti-UCP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Glut4 (Chemicon International, Inc., Temecula, CA); and anti-phospho-akt and anti-phospho-MAPK (New England BioLabs, Beverly, MA). Protein A-Sepharose was purchased from Amersham Biosciences (Piscataway, NJ), and [γ-32P]ATP was from PerkinElmer Life Sciences (Boston, MA). Phosphoinositide was isolated from Avanti Polar-Lipids, Inc. (Alabaster, AL), polyvinylidene difluoride membrane was from Fisher Scientific (Pittsburgh, PA), thin-layer chromatography plates were from VWR (Bridgeport, NJ), and electrophoresis supplies were from Bio-Rad Laboratories (Hercules, CA). Trogilizone was a gift from Warner-Lambert Co. (Ann Arbor, MI). All other supplies, unless indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Isolation and Culture—Cells that were homoygous for a floxed allele of exon 4 of the insulin receptor (IRlox+) were used as controls for all recombinant experiments. Cells derived from normal adipocytes were isolated from newborn control IRlox and FIRKO mice by collagenase digestion as described previously (15). Pre-adipocytes were immortalized by infection with a pBABE retrovirus encoding SV40 T-antigen and selected with 2 μg/ml puromycin. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a 5% CO2 environment. For in vitro recombination of the insulin receptor, pre-adipocytes harboring a floxed allele of the insulin receptor (IRlox) were first plated at a subconfluent density. After 24 h, cells were infected with an adenovirus encoding cre recombinase at a titer of 10⁹ plaque-forming units. After 1 h the viral supernatant was replaced with culture medium. Individual colonies were selected for IR recombination using PCR with primers 1 and 2, and viral supernatants were collected 48 h after transfection.

Differentiation of the Cells—Cells in which the floxed allele of exon 4 of the insulin receptor had been deleted by recombinase at a titer of 10⁹ plaque-forming units were plated on coverslips in 10-cm plates. After the induction phase (day 0), cells were returned to differentiation medium supplemented with 5 μg/ml of insulin. At this time, experimentally differentiated cells were morphologically distinct from non-stimulated cells and had visible fat droplets. At day 3, following the induction of adipogenesis, the cells were fixed and stained with Oil Red O.

Oil Red O Staining—Dishes were washed with ice-cold phosphate-buffered saline and fixed with 10% buffered formaldehyde for at least 16 h at 4°C. Cells were then washed twice with cold water and air-dried. Staining was performed using Oil Red O solution (5 g/liter in isopropl alcohol) washed three times with water, and visualized.

Plasmids and Retroviral Infection—The PPARY retroviral expression vector has been described previously (1). Coding sequences for the individual splice isoforms of the human IR either containing or lacking exon 11 (kindly provided by Ingo Leibiger, Karolinska University, Stockholm, Sweden) were cloned into pBABE-helo or -hgyro vectors. Subconfluent dNX packaging cells were transfected by Lipofectamine™ reagent (Invitrogen, Carlsbad, CA) with 10 μg of retroviral vector, and viral supernatants were collected 48 h after transfection. Control or IRKO pre-adipocytes were infected with Polybrene (4 μg/ml)-supplemented virus for 48 h and then placed in selection medium containing either the bleomycin analog neos (250 μg/ml) or hygromycin (200 μg/ml, Invitrogen).

RNA Extraction and Semi-quantitative RT-PCR—Total RNA was isolated using the single-step method of Chomczynski and Sacchi (17). To analyze gene expression by PCR, 1 μg of total RNA was primed with oligo(dT) and reverse transcribed using the first-strand cDNA synthesis kit (Invitrogen) to synthesize cDNA. The samples were diluted 5-fold, and 5% of the total volume was used for subsequent PCR. Primers and PCR conditions are as follows: mouse IR exon 4 primer 1, 5'-ACGTGCAGGAACCTGATGCC-3', primer 2, 5'-GCTGA-TACCCAGGATGAGGCAC-3'; mouse IR exon 11 primer 1, 5'-ATGAGATTCCAGCAGCAGC-3', primer 2, 5'-CTGTCGAGCACAATTGGA-3'; human IR exon 11 primer 1, 5'-ACCGAGTGATTGGACGAG-3', primer 2, 5'-ACCAATGATGGATGAGCAGG-3'; primer 2, 5'-TCCCCAGTCTGTCGATGAGC-3'; Glut4 primer 1, 5'-GGGACAATGTGATGAGC-3'; Glut4 primer 2, 5'-ACCAATGATGGATGAGCAGG-3'; PPARα primer 1, 5'-GAATACCAAAGTCTGATCAGA-3'; primer 2, 5'-CCTACCGAGCTGAACGGCT-3'.

RESULTS

1. Alternative Splicing of the Insulin Receptor Is Regulated During Brown Fat Adipogenesis—Insulin receptor-deficient brown pre-adipocytes for these studies were derived in two independent fashions. In one, brown fat precursor cells from the interscapular fat pad of the fat-specific insulin receptor knockout (FIRKO) mice were directly immortalized from neonatal mice using a retrovirus expressing the SV40 T-antigen. In these FIRKO cells, cre recombinase is driven by the adipose-specific promoter aP2. While this promoter is mainly expressed in mature adipocytes, low levels of aP2 mRNA expression have been observed in pre-adipocytes before the onset of adipogenesis (19). A low level of cre recombinase expression driven by this promoter was sufficient to recombine the insulin receptor alleles in these pre-adipocytes. Alternatively normal differentiating cells in which both alleles of exon 4 of the insulin receptor were flanked with loxP sites (IRlox+/+) were immortalized using SV40 T-antigen then infected in vitro with an adenovirus encoding cre recombinase. Individual colonies containing the recombinated allele (IRKO) were selected and propagated. Cells derived by both methods behaved similarly, and we have focused on cells derived by in vitro recombination for simplicity.

2. The Role of the Insulin Receptor in Differentiation of the Cells—Cells derived by both methods behaved similarly, and we have focused on cells derived by in vitro recombination for simplicity. Fig. 1 shows a representative IRKO pre-adipocyte cell line in which exon 4 of IR has been deleted. A 480-bp PCR product was present in IRlox+ cells, whereas a smaller 220-bp product was observed in IRKO cells, confirming that recombination had occurred. To test IR expression, individual splice isoforms of the human IR were introduced into IRKO cells by retroviral infection. While IR mRNA was still present in IRKO cells, no mature protein was detected in these cells, and reconstituted levels of IR are higher than that seen in control cells (Fig. 1, bottom panel).
Reconstitution of IR in IRKO Cells Fails to Restore Differentiation

To determine whether the re-expressed IR isoforms were functional proteins, control, IRKO, and IR-reconstituted KO pre-adipocytes were stimulated with insulin, and phosphorylation of downstream targets was analyzed (Fig. 3). IR protein expression was very low in control IRlox pre-adipocytes, but was undetectable in IRKO cells. IR-reconstituted KO cells showed dramatically increased IR protein levels and a markedly increased level of IR tyrosine phosphorylation following insulin stimulation. Insulin was also able to stimulate phosphorylation of AKT in control and IR-reconstituted KO cells, but not in IRKO cells. Therefore, IR is required for insulin-stimulated AKT phosphorylation. Surprisingly, despite the absence of insulin receptors, insulin was able to induce phosphorylation of MAPK in IRKO cells, although phosphorylation was slightly enhanced in cells re-expressing IR. Because IRKO cells lack insulin receptors, this increase in MAPK phosphorylation in IRKO pre-adipocytes most likely occurs via insulin action through the IGF-I receptor. This result was not due to receptor compensation, because IGF-I receptor levels were not different between the cell lines (data not shown). Both splice isoforms of IR (hIRa and hIRb) were equitant in their ability to activate downstream targets.

Phosphorylation of IRS-1 and IRS-2 during Adipogenesis

A number of proteins implicated in adipogenesis were examined during differentiation. To ensure that the re-expressed IR isoforms were functional during differentiation, IRS-1 and IRS-2 were examined in control, IRKO, and IR-reconstituted KO cells (Fig. 3). Because hIRa and hIRb signaled similarly to IR in these events, tyrosine phosphorylation of IRS-1 and IRS-2 were examined in control (IRlox), IRKO, and IR-reconstituted KO cells during the differentiation protocol. Because hIRa and hIRb signaled similarly to IR in these events (see Fig. 3), only results for cells expressing hIRa are shown for simplicity. Similar to mRNA levels, protein expression of IRS-1 and IRS-2 was low in control pre-adipocytes but was increased in mature adipocytes (Fig. 4). IRKO cells failed to express mature IR protein. IR-reconstituted KO cells showed an abundant increase in expression of IR in pre-adipocytes (day 0). Although these levels were maintained throughout differentiation, the absolute level of IR was only ~3-fold higher at day 6 compared with control adipocytes.

Following induction of differentiation, tyrosine phosphorylation of IR was elevated at day 2 in control cells. At days 4–6 of differentiation, levels of IR tyrosine phosphorylation declined even though total IR protein levels were elevated. As expected, IRKO cells showed no increase in IR tyrosine phosphorylation. In IR-reconstituted KO cells tyrosine phosphorylation of IR was faintly detected at day 0 but was dramatically increased at day 2 after induction of differentiation. Following the pattern of IR tyrosine phosphorylation, both IRS-1 and IRS-2 were tyrosine-phosphorylated at day 2 of differentiation in control cells. This phosphorylation was absent in IRKO cells and dramatically reduced in IR-reconstituted KO cells despite the high levels of IR tyrosine kinase activity.

Reconstitution of IR Expression in IRKO Cells Restores PI3K Activation and MAPK Dephosphorylation—Inhibition of PI3K activity by pharmacological inhibitors or expression of dominant-negative subunits disrupts adipocyte differentiation (1, 4), indicating PI3K activity is essential for the adipogenic program. To determine whether PI3K activation was directly coupled to differentiation, PI3K activity in phosphotyrosine immunoprecipitates was analyzed in the three cell lines. Similar to the pattern of IRS-1 and IRS-2 tyrosine phosphorylation, PI3K activity was induced ~4-fold at day 2 and then returned to basal levels at days 4 and 6 in control cells (Fig. 5A). There was no induction of PI3K activity in IRKO cells. In IR-reconstituted cells, PI3K activity was dramatically increased at day 2.
KO cells basal PI3K activity was higher, and there was a further induction at day 2 of differentiation. Thus, although the insulin receptor appears to be required for the PI3K activation observed during differentiation, activation of PI3K, in the presence of an inappropriate level of IR, is not sufficient to drive the adipogenic program.

To investigate signaling further downstream of IR, IRS, and PI3K, phosphorylation of AKT and MAPK was examined. In control cells, there was a transient increase in AKT phosphorylation at day 2 of differentiation that paralleled PI3K activation (Fig. 5B, upper panel). Phosphorylation of AKT was absent in IRKO cells. IR-reconstituted KO cells showed an increase in AKT phosphorylation at day 0, which declined during differentiation. MAPK activity has been associated with inhibition of differentiation (21, 22), and in accordance with this, MAPK phosphorylation was elevated in control pre-adipocytes (day 0) but then decreased during differentiation (Fig. 5B, lower panel).
Panel). By contrast, MAPK phosphorylation in IRKO cells was high and remained elevated throughout the culture period, correlating with the inability of these cells to differentiate. Phosphorylation of MAPK in IR-reconstituted KO cells resembled the pattern observed in control cells, i.e. phosphorylation was elevated in pre-adipocytes and declined at the later days of the differentiation program. However, as noted above, these cells failed to differentiate.

PPARγ Expression and Activation Partially Restores Differentiation in IRKO Cells—The finding that IR-reconstituted KO cells were similar to control cells in their ability to alter differentiation-dependent signaling such as activation of PI3K and down-regulation of MAPK phosphorylation, but failed to differentiate as measured by oil red O staining or accumulation of adipogenic markers such as PPARγ and Glut4, suggested that the block in adipogenesis in these cells may occur at a later stage in differentiation.

To test this hypothesis, the adipogenic transcription factor PPARγ was introduced into IRKO cells via retroviral-mediated gene transfer. To enhance PPARγ activation, some experiments were also performed with the addition of the PPARγ agonist troglitazone. Differentiation was measured by the induction of the adipogenic proteins PPARγ, Glut4, FAS, and C/EBPα (p42 and p30 products). As described above, fully differentiated control cells showed high expression of all the measured adipogenic markers (Fig. 6, lane 1). Addition of PPARγ or troglitazone in control cells exhibited no significant changes in protein expression, presumably due to the natural differentiation potential of these cells (Fig. 6, lanes 2–4). In contrast with the results in IRS-1 knockout cells (1), expression of PPARγ or its activation by troglitazone only slightly improved the ability of IRKO cells to differentiate as measured by a modest increase in the levels of endogenous PPARγ, Glut4, and FAS (Fig. 6, compare lanes 6 and 7 to lane 5). The combination of PPARγ expression with activation by troglitazone further enhanced differentiation in IRKO cells (Fig. 6, compare lane 8 with lanes 6 and 7). However, under these conditions...
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Because IRKO cells reconstituted with IR failed to differentiate but exhibited some differentiation-dependent signaling (see above), PI3K activation was examined in control cells that overexpress IR. As shown above, PI3K activity associated with phosphotyrosine increased ~4-fold at day 2 in control cells (Fig. 5C). Although basal PI3K activity was higher in cells overexpressing IR, a similar maximal level of activity was observed at day 2 of differentiation. The pattern of PI3K activation was paralleled by phosphorylation of AKT, a downstream target (Fig. 5D, upper panel). In cells overexpressing IR, AKT phosphorylation was initially high at the beginning of the differentiation program (days 0 and 2) but decreased thereafter. MAPK phosphorylation was also elevated in pre-adipocytes (Fig. 5D, lower panel). This phosphorylation was absent during the middle phases of the differentiation protocol but was increased again by day 6. Control cells that overexpress IR fail to differentiate but maintain insulin signaling through PI3K, AKT, and MAPK.

**DISCUSSION**

In the current study we have utilized SV40T-antigen-immortalized brown pre-adipocytes isolated from control and IRKO mice to examine the role of the insulin receptor in adipocyte differentiation. In contrast to the pattern in models of white adipogenesis, such as 3T3-L1 cells, the expression level of the insulin receptor is very low in brown pre-adipocytes and is dramatically induced during adipocyte maturation. In addition, insulin receptor mRNA is also induced during differentiation such that the A isoform (~exon 11) in IRKO cells reconstituted with IR fails to differentiate in vivo, thus HepG2 cells cultured at low density exhibit a fetal phenotype and express predominantly the B isoform of the insulin receptor, whereas HepG2 cells cultured to confluence in the presence of dexamethasone display an adult phenotype and express the B isoform of the insulin receptor (23). Likewise, in rat liver in vivo, IRB expression increases from 60% in fetal liver to 95% in adult liver (12). In addition to dexamethasone, insulin itself may regulate splicing of the insulin receptor gene. For example, FAO hepatoma cells treated with insulin show a time-dependent decrease in IRA with a relative increase in IRB (24). Therefore, both differentiation itself and the hormonal milieu used during differentiation of adipocytes may regulate the alternative splicing of the insulin receptor.

Expression of either IRA or IRB restores insulin signaling in IR-deficient pre-adipocytes. In contrast to observations in pancreatic β cells (25), both receptors are equally capable of initiating phosphorylation of AKT, MAPK, or p70S6K (data not shown) in response to insulin. However, we were unable to examine the individual contribution of each isoform in mature adipocytes where expression of both receptors occurs naturally.

Using standard differentiation protocols, we observed that insulin receptor-deficient brown pre-adipocytes failed to differentiate as measured by the lack of lipid accumulation or the expression of the adipogenic markers PPARγ, FAS, and Glut4. The requirement for the insulin receptor in adipocyte differentiation in vitro is supported by gene dosage studies performed in 3T3-L1 cells. 3T3-L1 fibroblasts with a somatic inactivation of one allele of the insulin receptor showed a 50–70% reduction in IR levels and exhibited impaired differentiation with only 30–50% of cells displaying morphological evidence of adipogenesis (26). Although insulin receptor levels are low in pre-adipocytes, this minimal expression is required to initiate the
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adipogenic program. The failure of IRKO cells to differentiate also resembles defects seen in IRS-1-deficient cells, reiterating the importance of insulin signaling in brown fat adipogenesis.

Even though control or IRKO cells overexpressing IR fail to terminally differentiate, some insulin receptor- and differentiation-dependent signaling is preserved in these cells. For example, PI3K activity peaks at day 2 in control cells. Although PI3K activity is absent in IRKO cells, IR reconstitution restores a similar pattern of PI3K activity after the hormonal induction of adipogenesis, suggesting that the insulin receptor is required for this event. PI3K activity is also maintained in control pre-adipocytes that overexpress IR. Previous data has suggested that PI3K activity during the induction phase is essential for adipogenesis in both white and brown fat cell models. Our data would suggest that, although required, PI3K activity is not sufficient to drive adipocyte differentiation.

Previous studies have shown that high MAPK phosphorylation correlates with the inability to differentiate, and overexpression of MAPK or its upstream activator MEK1 inhibits 3T3-L1 differentiation, possibly by reducing PPARγ activity (22, 27). In control pre-adipocytes, MAPK phosphorylation is high then declines with differentiation. IRKO cells, which fail to differentiate, also fail to attenuate MAPK phosphorylation. However, IR-reconstituted cells and control cells overexpressing IR exhibit a decrease in MAPK phosphorylation during the differentiation program similar to control cells but show similar defects in adipogenesis. These data suggest that the classic insulin-dependent signaling induced during the early phases of differentiation can be uncoupled from the production of mature adipocyte phenotype.

Unlike IRS-1 reconstitution in IRS-1 KO cells, expression of the insulin receptor in IRKO cells fails to parallel lipid or induce adipogenic program (1). IR-reconstituted KO pre-adipocytes fail to generate lipids or induce adipogenic program. This appears to be a unique property of the insulin receptor in the pre-adipocyte stage. This hypothesis is validated by the fact that control pre-adipocytes overexpressing IR also fail to differentiate. The decreased levels of IR re-introduced in IRKO cells are unable to recruit the balance between IR and IGF-IR required for normal differentiation (22).

Insulin and IGF-I receptors are high-affinity homologous receptors, each composed of two αβ-heterodimers linked by disulfide bonds (28, 29). Individual αβ-heterodimer subunits from IR and IGF-IR can also combine to form hybrid receptors (30). Previous studies have shown that both IGF-I and insulin are able to induce adipogenic and thermogenic genes in brown adipocytes (31). Fetal brown adipocytes possess a number of high affinity IGF-I binding sites and express both IGF-I and IGF-IR mRNA during development (32). As noted above, while insulin receptor expression increases during differentiation, overexpression of IR inhibits differentiation. In brown pre-adipocytes and 3T3-L1 fibroblasts, IR levels are low and IGF-IR is the primary signaling receptor (33). With the induction of differentiation, IR expression increases dramatically such that it is the predominant receptor in mature adipocytes. Hybrid receptor formation rises in parallel with the increase in insulin receptor levels. High IR expression would increase the number of binding sites for insulin through classic insulin receptors but place more of the IGF-I receptors in hybrid complexes. We hypothesize that these hybrids are like the insulin receptor in that they are unable to support differentiation. Thus, the increase in the insulin signaling pathway indirectly alters the response to the IGF-I receptor, which adversely affects cell growth or differentiation. Indeed, Chinese hamster ovary cells overexpressing the insulin receptor are more sensitive to insulin but become resistant to IGF-I, although the levels of IGF-IR remain unchanged, suggesting that high levels of IR impair IGF-I signaling (34). Taken together, these data suggest that abnormal expression of IR in pre-adipocytes might disrupt crucial IGF-I signaling required for adipogenesis. Therefore, a correct balance of IR and IGF-IR must be tightly controlled at all stages of differentiation. Depletion or inappropriate overexpression of the insulin receptor early in the process impairs brown adipocyte differentiation. Thus, the increase in insulin receptor later in adipogenesis may be important for both insulin regulation of metabolism and the normal differentiation of this cell type.

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