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Regulation of Proteins Involved in Insulin Signaling Pathways in Differentiating Human Adipocytes

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In the present study we have examined the proteins involved in the insulin signaling cascade during and after differentiation of human adipocyte precursor cells and their correlation with glucose uptake. The differentiation of human adipocytes was characterized by a two- to threefold stimulation of glucose transport in response to insulin and a marked increase protein expression for the insulin receptor, IRS-1, GLUT-4, PI 3-kinase, and PKB, with respect to undifferentiated cells. In contrast, there were small changes in the protein expression of IRS-2, and no changes in PKC zeta and MAP kinases, although basal MAP kinase activity and GLUT-1 protein were reduced during differentiation. In conclusion, there are quantitative differences in the regulation of IRS-1 and other proteins during differentiation which may contribute to more efficient insulin signaling leading to glucose uptake in mature fat cells. Alterations in this pattern may reflect or contribute to an insulin-resistant state. © 2000 Academic Press

Key Words: **human adipocytes; differentiation; insulin signaling proteins; glucose transport.**

Traditionally, adipocytes have been implicated in lipid homeostasis and energy balance but it is now recognized that they play a central role in metabolism, being a target for insulin action. The ability of insulin to promote glucose storage in muscle and fat is crucial to the maintenance of glucose homeostasis and an impairment in the ability of insulin to stimulate glucose uptake contributes to the development of insulin resistance and type 2 diabetes (1).

Insulin action is initiated through hormone binding to cell surface insulin receptors which activates the protein kinase associated with the beta subunit. The stimulation of the receptor kinase activity induces the phosphorylation of insulin receptor substrates (IRS) which allows them to interact with and recruit SH2-domain-containing proteins including phosphatidylinositol (PI) 3-kinase (2). Activation of PI 3-kinase triggers an insulin-stimulated protein kinase cascade including PDK1 and 2 (phosphatidylinositol 3,4,5-trisphosphate-dependent protein kinase) (3) that activate the serine/threonine kinase PKB (protein kinase B) and PKCs zeta/lambda that have been implicated in the insulin signaling pathway leading to the translocation of GLUT-4 to the plasma membrane and glucose uptake (4, 5).

The molecular events associated with preadipocyte differentiation have been mostly studied in 3T3-L1 cells (6–8). Treatment of preadipocytes with inducers of differentiation stimulates a rapid and transient increase in C/EBPβ and C/EBPδ, which in turn mediates the transcriptional activation of peroxisomal proliferator-activated receptor γ (PPARγ) and subsequent induction of C/EBPα expression (9–11). Together, C/EBPα and PPARγ activate the transcription of genes involved in creating and maintaining the adipocyte phenotype such as ap2, stearoyl coenzyme A, desaturase I, glucose transporter (Glut)-4 and leptin (12, 13). However, little is known about the regulation of insulin signaling proteins and their isoforms during the differentiation of human adipocytes.

In the present study, we have examined several of the proteins involved in the insulin signaling cascade before and after differentiation of human adipocyte precursor cells and their correlation with glucose uptake.

MATERIALS AND METHODS

Cells. Human adipocyte precursor cells were from Zen-Bio, Inc. (Research Triangle Park, NC). They were obtained from 5 female patients, age range 25–52 and with a BMI range of 22–27. Preadipocytes were cultured in DMEM high glucose (growth medium) containing 10% Fetal Bovine Serum and 1X Antibiotic/Antimycotic (GIBCO, Rockville, MD). Once the cells reached confluence, they were induced to differentiate by the addition of induction medium (growth medium containing 0.4 μM insulin, 0.25 μM dexamethasone, 0.5 mM MIX (Sigma, St. Louis, MO) and 1 μM rosiglitazone).

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After 3 days, the medium was changed and cells were fed every 2 to 3 days with growth medium. Cells were lysed at Day 0 (confluence) and at 14 days after induction of differentiation.

Glucose transport. Human adipocyte precursor cells were grown in 96-well plates and serum-starved for 2 h at 37°C, after which they were incubated in DMEM (without glucose) for an additional 30 min prior to incubation with or without insulin for 30 min. The assay was initiated by adding 2-deoxy-D-[^{3}H]glucose (130 μCi/sample, 0.2 mmol) and was terminated 45 min later by washing the cells twice with PBS. Cells were then solubilized and the incorporated radioactivity was determined by scintillation counting.

Immunoblotting. Cells were lysed in 0.4 ml of lysis buffer containing 25 mM Tris · HCl (pH 7.4), 0.5 mM EGTA, 25 mM NaCl, 1% Nonidet P-40, 1 mM Na3VO4, 10 mM NaF (Sigma, St. Louis, MO), 0.2 mM leupeptin, 1 mM benzamidine, and 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Calbiochem, La Jolla, CA) and rocked for 40 min at 4°C. Detergent-insoluble material was sedimented by centrifugation at 12,000*g* for 10 min at 4°C. Cell lysate proteins (50 μg of protein) were separated by SDS/PAGE on 7.5% gels (Bio-Rad, Hercules, CA). Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% milk. The blots were probed with various primary antibodies as follows: anti-IRS-1 C-terminal, anti-IRS-2, anti-p85 (whole antiserum), 4G10 anti-phosphotyrosine antibodies, anti-MAPK, anti-PKB (both PKBα and PKBβ), and anti-PKBβ antibodies (Upstate Biotechnology, Lake Placid, NY); anti-insulin receptor (Transduction Laboratories, Lexington, KY); anti-p110α and β, anti-PKCζ (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Active MAPK (Promega, Madison, WI); anti-GLUT-4 antibodies (Chemicon, Temecula, CA) according to the recommendations of the manufacturers or anti-GLUT-1 antibodies, a kind gift from Dr. Sam Cushman, NIH. The proteins were detected by enhanced chemiluminescence and horseradish peroxidase-labeled secondary antibodies (Amersham). The intensity of the bands was quantitated with a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Insulin-Stimulated Glucose Transport Increases with Adipocyte Differentiation

Human preadipocytes were cultured and differentiation was induced by the addition of dexamethasone, insulin, MIX, and rosiglitazone as described under Materials and Methods. This differentiation scheme resulted in more than 90% of preadipocytes accumulating triacylglycerol after 10 days of culture and by day 14 those droplets became bigger and unilocular (Fig. 1).

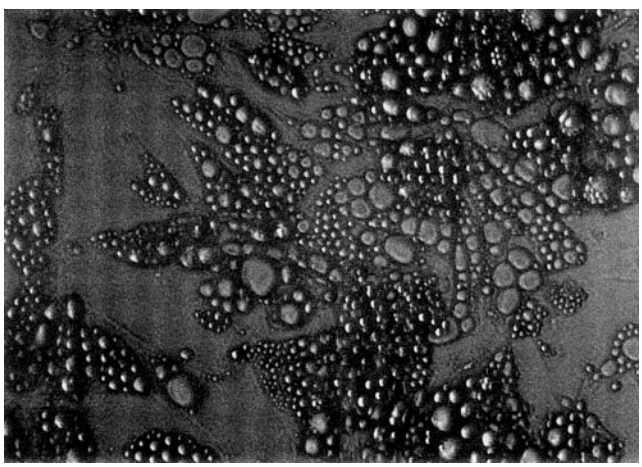


FIG. 1. Human adipocytes after 14 days of induction of differentiation. Photograph is of the cells without fixing or staining. Magnification, 400×.

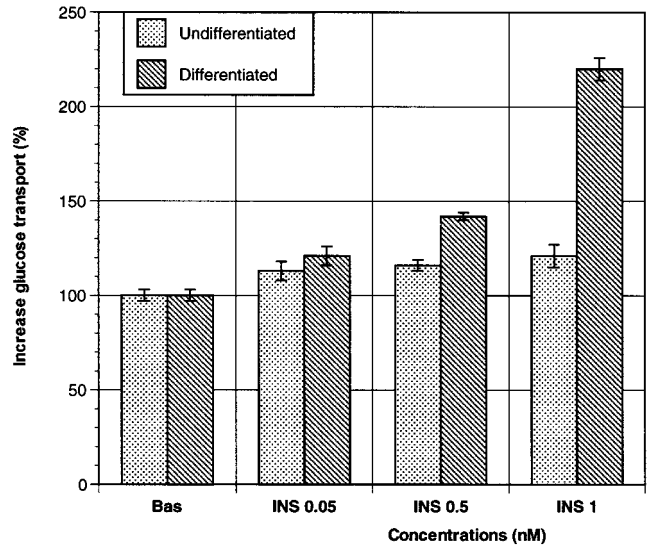
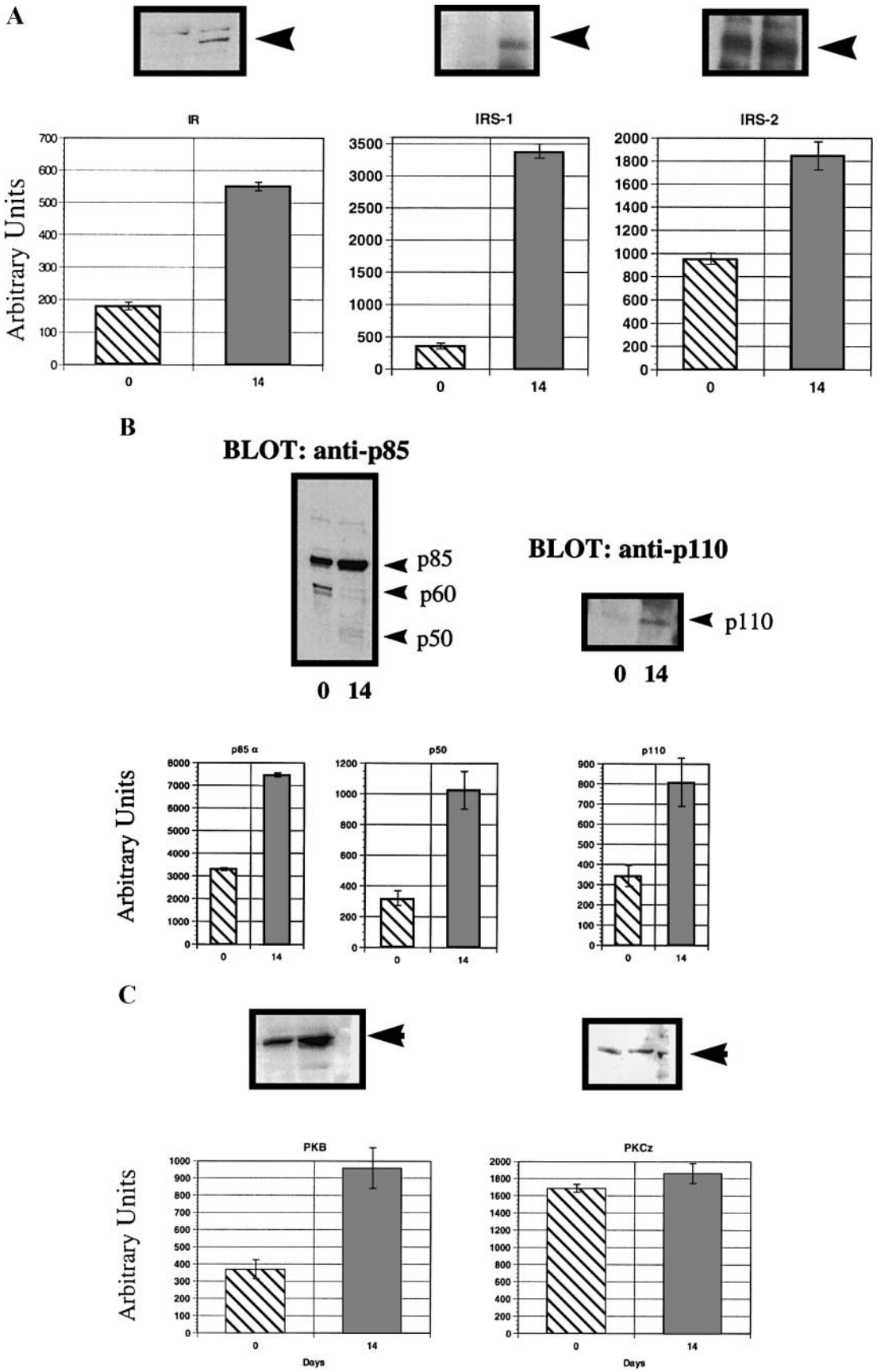


FIG. 2. Glucose uptake was measured in confluent and differentiated human fat cells incubated for 30 min with different concentrations of insulin as shown. At that time, 2-deoxy-D-[^{3}H]glucose was added and the incubations were continued for 45 min as described under Materials and Methods. Results are means ± SE of four separate experiments performed in triplicates.

At confluence, undifferentiated cells did not respond to insulin at any of the concentrations. In contrast, maximal physiological concentration of insulin (1 nM) stimulated glucose uptake by two- to threefold in differentiated adipocytes, very similar to the effect seen previously using fresh human adipocytes (14) (Fig. 2).

Glucose Transport Correlates to Expression of Proteins Involved in Insulin Signaling

To examine the expression of proteins involved in insulin action during differentiation of human adipocytes, lysates were prepared from confluent and differentiated adipocytes and proteins were detected by immunoblotting with the specific antibodies. The differentiation of human adipocytes was characterized by a 3 fold increase of insulin receptor and a 10-fold increase of IRS-1 protein, while IRS-2 protein was increased by only 2-fold (Fig. 3A). The regulatory subunit of PI 3-kinase, p85α and its splicing variant p50, increased by 2- to 3-fold, while the p110 catalytic subunit increased by 2-fold with differentiation. Interestingly, there was a doublet at 60,000 MW (p60) recognized by the p85 whole antiserum that decreased with the differentiation (Fig. 3B), while p55α, another p85α splicing variant, could not be detected in these cells.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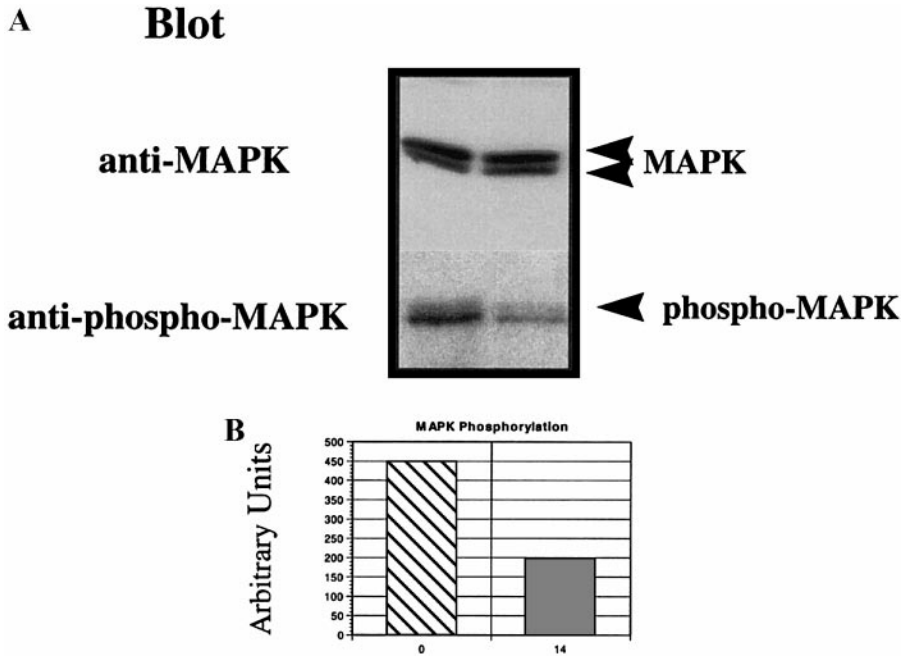

FIG. 4. MAP kinase phosphorylation and protein levels before and after differentiation. Lysates were obtained from human fat cells at confluence (Day 0) or after 14 days of differentiation (Day 14). Following separation (7.5% SDS–PAGE), the proteins were immunoblotted with MAP kinase antibodies and anti-phospho MAPK antibodies as described. (A) Immunoblot of a representative experiment; (B) quantification of the bands by laser densitometry. The experiment was repeated three times with similar results.

Recent work suggests that PKB and PKCζ/λ are implicated in the insulin-induced glucose transport in adipocytes (4, 5). Therefore, we first performed experiments to examine which isoforms were present in human adipocytes. PKBα and β were present in those cells, similar to data from fresh human adipocytes (15) but only PKCζ, and not PKCλ was detected (not shown). PKBα and PKBβ, detected by an antibody that recognized both isoforms (16), increased by approximately 3-fold during differentiation. The same results were obtained using antibodies that recognized only the PKBβ isoform (not shown). In contrast, no changes were found with PKCζ (Fig. 3C). In addition, we checked whether the mitogen-activated protein (MAP) kinase pathway changed during differentiation. No changes in MAP kinase protein levels were detected before and after differentiation, although there was a significant decrease in basal MAP kinase activation in fully differentiated adipocytes (Fig. 4).

Finally, protein expression of glucose transporters that are involved in glucose transport in insulin-target tissues were determined by immunoblotting. As expected, GLUT-4 increased by 40-fold while GLUT-1 decreased during differentiation (Fig. 5).

DISCUSSION

This study provides the first detailed assessment of the protein expression during human fat differentiation of some of the most important proteins involved in insulin signaling leading to glucose transport.

Insulin binding to its cell surface receptors activates the intrinsic tyrosine kinase activity of the insulin receptor and stimulates the phosphorylation of insulin receptor substrate proteins allowing them to interact with and recruit SH2-domain-containing proteins including PI 3-kinase. Products of PI 3-kinase recruit serine kinases to the plasma membrane, including PDK1 and 2, that activate the serine/threonine kinase PKB which has been implicated in the insulin signaling pathway leading to glucose transport, protein synthesis, glycogen synthesis, cell proliferation, and cell survival in various cells and tissues (17, 18).

We have found that glucose transport increased in response to insulin during differentiation of human adipocytes and that this was correlated with increased levels of proteins involved in the insulin signaling pathway. A greater increase in IRS-1 and GLUT-4

FIG. 3. Regulation of proteins involved in insulin signaling before and after human fat differentiation. Lysates were obtained from human fat cells at confluence (Day 0) or after 14 days of differentiation (Day 14). Following separation (7.5% SDS–PAGE), the proteins were immunoblotted with (A) anti-insulin receptor, anti-IRS-1 and anti-IRS-2 antibodies (B) anti-p85 (PI 3-kinase) whole antiserum and anti p110 antibodies (C) anti-PKB α and anti-PKCzeta antibodies. Results are represented as $\pm$SE arbitrary densitometric units (n = 4). The experiment was repeated four times with similar results.

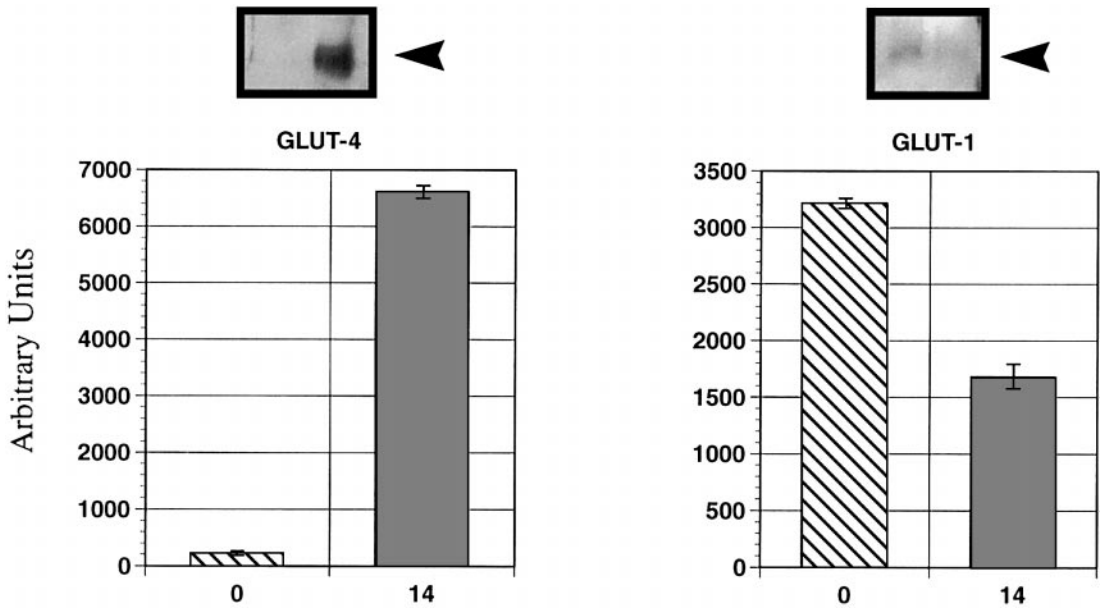

FIG. 5. Glucose transporters protein levels before and after human fat differentiation. Lysates were obtained from human fat cells at confluence (Day 0) or after 14 days of differentiation (Day 14). Following separation (7.5% SDS–PAGE), the proteins were immunoblotted with antibodies against GLUT-1 and GLUT-4. Results are represented as ±SE arbitrary densitometric units ($n = 4$). The experiment was repeated four times with similar results.

proteins was seen, suggesting an important role of these proteins in the development of insulin sensitivity in human fat cells. Since IRS-1 is a primary substrate for the insulin receptor, it is also a good candidate for variable regulation in insulin resistant states. For example, a defect in IRS-1 would be expected to lead to a decrease in insulin action and tissue insulin resistance. We have recently examined the expression and function of IRS-1 and IRS-2 in adipocytes from healthy and diabetic individuals (19, 20). Fat cells from type 2 diabetic patients showed a reduced expression of IRS-1 and GLUT-4 proteins leading to a decrease in insulin-stimulated glucose uptake and insulin resistance *in vivo* (19), while IRS-2 was unchanged. In fat cells from healthy individuals, IRS-1 is the main docking protein for binding and activation of insulin-stimulated PI 3-kinase; IRS-2 is also functional but requires a higher insulin concentration to achieve comparable binding of PI 3-kinase (19). Therefore, an increase of IRS-1 protein during differentiation is likely to be important for the development of insulin sensitivity in human adipocytes.

A significant increase in the insulin receptor, PI 3-kinase (p85 and p110) and PKB protein levels was also detected. Activation of PI 3-kinase is essential for aspects of insulin-induced glucose metabolism, including translocation of GLUT-4 to the cell surface and glycogen synthesis (21–23). The enzyme exists as multiple isoforms, owing to the differential expression and dimerization of multiple regulatory subunits (p85α and its splicing variants, e.g. p50α) and one of two widely distributed isoforms of the p110 catalytic subunit: p110α or p110β (24). During differentiation of 3T3-L1 cells into adipocytes, p110β was reported to be upregulated (25), while in human adipocytes p110α, but not p110β (not shown), was increased during differentiation. Interestingly, we could detect one of the p85 splice variants, p50, but not p55, in human adipocytes and it was increased during differentiation. Both p50 and p55 are splice variants containing identical N-SH2 domains and would therefore be expected to cross-react with the p85 antisera with a similar affinity as for p85 itself. Interestingly, it was found that p85α knock-out mice were hypoglycemic and they had an increase in insulin-stimulated glucose transport that correlated with an increase in the splicing products (26), suggesting an important role of these isoforms in the development of insulin sensitivity. In agreement with our study, another report also showed that differentiation of 3T3-F442A cells was characterized by a 13-fold increase in the insulin receptor, a 9-fold increase in IRS-1, and a 3-fold increase in p85 subunit of PI 3-kinase (27).

Another interesting observation was that only PKB increased during differentiation of human fat cells, in agreement with other reports utilizing 3T3-L1 cells (16), while PKCζ remained the same, suggesting the importance of PKB in the insulin sensitivity of mature adipocytes.

GLUT-4 is well known to play an important role in maintaining glucose homeostasis in mammals. Downregulation of its expression in adipocytes is a

universal feature of insulin-resistance states. A number of transgenic studies show that overexpression of GLUT-4 in either muscle or adipose tissue alone or together (28, 29, 30) enhances glucose tolerance and insulin-dependent glucose uptake *in vivo.* Elevating the level of GLUT-4 expression in these tissues prevents insulin resistance in normal mice (31). In contrast, high level of GLUT-1 seem to be correlated with insulin resistance. Transgenic mice that overexpress GLUT-1 in skeletal muscle develop insulin resistance (32). In this study, a large increase in GLUT-4 protein and a decrease in GLUT-1 were observed during differentiation of human preadipocytes. This is in agreement with a previous study which found that when human adipocytes acquired the adipocyte phenotype, there was an increase in the stimulation of glucose transport by insulin compared to basal states, and this was correlated with an increase of GLUT-4 protein levels and a decreased amount of GLUT-1 protein (33).

To check whether the mitogenic insulin signaling pathway was altered during differentiation, MAP kinase protein and activity were measured. MAP kinase activity in the basal state decreased in mature fat cells although protein levels were unchanged. This is an important observation since MAP kinases have been implicated in the development of insulin resistance by having a role in the serine phosphorylation of IRS-1 (34) and PPAR γ inactivation (35). Thus, activation of MAP kinases may inhibit adipogenesis and suppression of their activation may be necessary for the development of insulin sensitivity in mature fat cells.

In conclusion, regulation of expression of IRS-1, GLUT-4, and other proteins involved in insulin signaling during differentiation is associated with full development of insulin-stimulated glucose uptake in mature human adipocytes. We propose that alterations of this pattern may lead to insulin-resistant states.

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