Hyperinsulinemia Induces Insulin Resistance on Glucose and Lipid Metabolism in a Human Adipocytic Cell Line: Paracrine Interaction with Myocytes

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Context: Adipocytes release a variety of factors which deregulation could provide the basis for complications such as insulin resistance, an early defect on the onset of type 2 diabetes. Such insulin resistance can initially be overcome by compensatory hyperinsulinemia, but the prolonged presence of the hormone can be detrimental for insulin sensitivity.

Objective: The objective of the study was to dissect the molecular mechanisms that may regulate hyperinsulinemia-induced insulin resistance in a human liposarcoma cell line and its paracrine interactions with a human rhabdomyosarcoma cell line.

Designs: We studied glucose uptake, lipolysis, insulin signaling, and secretion pattern at different days of adipocyte differentiation in the presence of insulin.

Results: Adipocytes differentiated for 14 d gain insulin sensitivity on glucose uptake and inhibition of lipolysis, but prolonged cultures develop an insulin-resistant state characterized by an increase in phosphatase and tensin homolog-deleted on chromosome 10 expression and defects in insulin signaling at the insulin receptor substrate-1/AKT level. The secretion pattern of nonesterified fatty acids, IL-6, adiponectin, leptin, and monocyte chemotactic protein-1 was in keeping with the changes in insulin sensitivity during differentiation. An inverse biphasic response was also observed in human myocytes when they were cultured with various adipocyte-conditioned media, although insulin resistance was detected earlier than in adipocytes. This behavior mimics hyperinsulinemia because insulin action was restored when adipocytes were cultured in the absence of the hormone. Pharmacological treatment of adipocytes with a liver X receptor agonist reestablishes insulin-stimulated glucose uptake, whereas treatment with a peroxisome proliferator-activated receptor-γ agonist restored the antilipolytic action of insulin.

Conclusions: Hyperinsulinemia deregulates adipocyte secretion pattern, producing insulin resistance in adipocytes and myocytes, a situation that can be ameliorated with nuclear receptor agonists. (J Clin Endocrinol Metab 93: 2866–2876, 2008)

Currently there is strong evidence that adipose tissue is not only an inert energy-storage depot, but it is also an endocrine organ. White adipose tissue expresses and secretes a variety of adipokines, such as adiponectin, resistin, and leptin, and proinflammatory cytokines, such as IL-6, monocyte chemotactic protein (MCP)-1 and TNF-α (reviewed in Refs. 1, 2), that can act at both the local and systemic level modulating insulin sensitivity. Insulin resistance is an important contributor to the pathogens...
genesis of type 2 diabetes (T2D), and obesity is a risk factor for its development, due in part to the fact that an altered secretion pattern, with increase in proinflammatory and decrease in anti-inflammatory factors is found in the obese state (3). When peripheral tissues, including muscle and fat, are exposed to these proinflammatory cytokines, they develop an insulin-resistant state (4). Such resistance can initially be overcome by a compensatory increase in insulin secretion from pancreatic β-cells, but the prolonged presence of the hormone can be detrimental for insulin sensitivity in several tissues, including β-cells, that fail the compensatory secretion and T2D occurs (5).

Insulin action in adipose tissue involves stimulation of glucose uptake and inhibition of lipolysis, metabolic effects dependent on the phosphatidylinositol (PI) 3-kinase and AKT pathway (6). The clearance of circulating glucose depends on insulin-stimulated translocation of the glucose transporter (GLUT)-4 to the cell surface. Although adipose tissue accounts for only a small fraction of insulin-dependent glucose disposal, fat-selective knockout glut4 gene mice show impaired glucose tolerance, suggesting that the functional integrity of this organ is crucial in regulating intermediate metabolism (7). Actually, resistance to insulin-stimulated glucose transport in adipose tissue is one of the earliest defects detected in insulin-resistant states. Moreover, visceral adiposity has been associated with increase of lipolysis, with subsequent release of nonesterified fatty acids (NEFAs) into the circulation, contributing to development of skeletal muscle insulin resistance. However, it is not fully understood whether inhibition of lipolysis by insulin is impaired in adipose tissue.

We previously investigated how TNF-α treatment induces a state of insulin resistance on glucose uptake in both murine myocytes and brown adipocytes. The mechanism found involves serine phosphorylation of insulin receptor substrate (IRS)-1 and up-regulation of protein-tyrosine phosphatase (PTP)-1B (8, 9). The aim of this work was to investigate insulin sensitivity in terms of glucose uptake and inhibition of lipolysis in an adipocyte cell line derived from a human liposarcoma (10). Moreover, we studied the secretion profile in prolonged cultures, when hyperinsulinemia-induced insulin resistance was detected. Furthermore, we analyzed the paracrine interaction of adipocyte-conditioned media with human myocytes.

Nuclear receptors comprise a superfamily of related proteins that act as transcription factors for target genes involved in glucose and lipid metabolism. These proteins are activated by naturally produced lipids as well as synthetic compounds; some of them display insulin-sensitizing effects and antiinflammatory properties (11). Accordingly, the effectiveness of different nuclear receptor agonists to overcome hyperinsulinemia-induced insulin resistance was finally evaluated in this work.

**Materials and Methods**

**Materials**

Insulin, BSA, TTNPB, phytanic acid, isoproterenol, rosiglitazone, free glycerol/triglyceride determination kit, and anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). Autoradiographic films and 2-deoxy-D-[1-3H]glucose were purchased from GE Healthcare (Rham, UK). Antibody against fatty acid synthase (FAS) was from Transduction Laboratories (Lexington, KY) and phosphorylated (P)-IRS1(Ser312) from Affinity BioReagents (Golden, CO). Antibodies against GLUT-1, GLUT4, IRS1, IRS2, c-ebi, PTP1B, protein phosphatase 2A (PP2A), Src homology-PTP2, and P-IRS1 (Tyr612) were from Millipore (Bedford, MA). Antibodies against total and phosphorylated ERK1/2 (Thr202/Tyr204), AKT (Ser473), c-jun NH2 terminal kinase (JNK)-1/2 (Thr183/Tyr185), and p38MAPK (Thr180/Tyr182) were from Cell Signaling (Beverly, MA); against phosphatase and tensin homolog-deleted on chromosome 10 (PTEN; sc-7974), p85α (z-8), insulin receptor (IR) β-chain (sc-09), lipoprotein lipase (LPL; H-53), phospholipase Cγ1 (sc-81), and caveolin-1 (sc-894) were from Santa Cruz (Palo Alto, CA). Antibodies against hormone-sensitive lipase (HSL), perilipin A/B, and apor protein 2 (aP2) were from Abcam (Cambridge, UK). All other reagents used were of the purest grade available.

**Cell culture and treatments**

The human cell line LiSa-2 was maintained in DMEM supplemented with 10% fetal serum and antibiotics at 37 °C and 5% CO2. To induce adipogenic differentiation, cells were cultured in serum-free DMEM/F12 (1:1) supplemented with 10 μg/ml transferrin, 15 mM NaHCO3, 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 1 mM insulin, 20 μM triiodothyronine, 1 μM cortisol, and antibiotics. For analysis of hyperinsulinemia effects, cells were maintained either in differentiation medium with insulin (normal medium) or without the hormone between 14 and 21 d of differentiation. To treat hyperinsulinemia-induced insulin resistance, cells were maintained in normal differentiation medium in the absence or presence of various nuclear receptor agonists for the last 7 d. Media were changed every 2 d. The human embryonal rhabdomyosarcoma RD cell line was used as a model of human skeletal muscle cells. This cell line, kindly provided by Dr. Nieves Olmo (Complutense University, Madrid, Spain), was grown in DMEM supplemented with 10% fetal serum and antibiotics, at 37 °C and 5% CO2. Before performing the different metabolic and signaling studies, both adipocytes and myocytes were cultured overnight in serum-free and low-glucose medium (5.5 mM glucose).

**Analysis of intracellular lipids**

Accumulation of triglycerides was visualized by staining the cells with Oil Red O or Nile Red dye (Sigma-Aldrich). Cells were fixed with formalin and stained. The triglyceride accumulation was assessed microscopically. OD for Oil Red O was quantified spectrophotometrically at 510 nm. Lipid content was also analyzed by measuring intracellular content by enzymatic method using triglyceride determination kit (Sigma-Aldrich).

**Immunoblot analysis**

Cellular proteins were submitted to SDS-PAGE, transferred to Immobilon membranes, and blocked (9). Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL-Plus) Western-blot protocol (GE Healthcare).

**Glucose transport and GLUT4 translocation assays**

Glucose uptake was measured during the last 10 min of culture (initial velocity conditions) by incorporation of 2-deoxy-D-[1-3H]glucose (11.0 Ci/mmol) as previously described (9). Results are expressed as picomoles of glucose per milligram of protein per 10 min. Cells were submitted to subcellular fractionation for plasma membrane and internal membrane isolation before immunoblotting with GLUT4, GLUT1, and caveolin-1 antibodies (9).

**Determination of lipolytic rate**

Rate of lipolysis was measured by following the rate of glycerol and NEFA release from the cells. Adipocytes were cultured overnight in serum-free medium prior stimulation. After washing, cells were incubated at 37 °C for 3 h, and glycerol released was assayed by enzymatic method.
using free-glycerol determination kit (Sigma-Aldrich). Results are expressed as nanomoles of glycerol per milligram of protein per 3 h. NEFA release was determined by an enzymatic colorimetric test (Wako Chemicals, Neuss, Germany) and was expressed as micromoles.

Preparation of adipocyte-conditioned media and measurement of adipokines release
LiSa-2 cells were cultured overnight in serum-free medium before obtaining the adipocyte-conditioned media. Human adipokines were determined using Luminex xMAP technology with multiplex immunoassays (LincoPlex) for simultaneous quantitative determination of adiponectin, MCP-1, leptin, TNF-α, resistin, and IL-6 (Millipore). The detection limits for adiponectin, MCP-1, leptin, TNF-α, resistin, and IL-6 were 80.3, 0.5, 27.4, 0.1, 4.5, and 0.10 pg/ml, respectively.

Real-time quantitative RT-PCR (QPCR) assays
DNase I-treated RNA was reverse transcribed into cDNA before performing the QPCR for leptin gene expression using the Taqman gene expression assays (9). The results are given as percentage over undifferentiated cells after normalizing mRNA to 18S rRNA expression.

Cell cycle analysis
DNA analysis was performed by flow-cytometry. Cells were trypsinized, counted, washed with PBS, and fixed with cold ethanol (70%). Then they were resuspended in PBS (10^6 cells/ml) treated with RNase (Roche Diagnostics, Indianapolis, IN) for 30 min at 37°C, stained with 0.05% of propidium iodide, and analyzed in the cytometer. The percentage of cells in the different phases of cell cycle and cell size was determined.

Data analysis
Results are presented as means ± SE from three to five independent experiments. Statistical significance was tested with the unpaired Student's t test or one-way ANOVA followed by the protected least-significant different test. P < 0.01 were considered significant.

Results
Modulation of insulin sensitivity through human adipocyte differentiation
LiSa-2 is a human cell line that retains the potential to undergo adipose differentiation, in which the components involved in insulin action have not been explored yet (10). We characterized the protein expression pattern on adipocytes at different stages of differentiation (Fig. 1, A and B). When examining the main elements of the insulin-signaling cascade, the expression of IR β-chain, IRS1, IRS2, and p85α-P13-kinase increased though the differentiation process. The expression of PTPIB and PTEN markedly increased on d 14 and 21 of differentiation without significant differences in PP2A, or SH-PTP2. Regarding proteins involved in glucose transport, an increase on GLUT1 expression was found from the beginning of the differentiation process, but c-cbl expression was not modified; meanwhile, GLUT4 increased on d 14. An increase in proteins involved in lipid metabolism, such as HSL, perilipins, LPL, ap2, and FAS was also observed on 14 and 21 d of differentiation, without changes in the expression of phospholipase Cγ1. Differentiation of LiSa-2 cells was also substantiated by accumulation of intracellular lipids (determined by staining with Oil Red O, Fig. 1C) and determination of triglyceride content (Fig. 1D).

Accordingly, we investigated glucose uptake and lipolysis through differentiation. Glucose uptake was stimulated by insulin only in 14 d differentiated cells, producing a maximal stimulation of nearly 2-fold (90%) at 10 nM insulin (Fig. 2A). In prolonged cell cultures (21 d), we detected a large increase (6-fold increase) of basal glucose uptake and a lack of stimulation with insulin at all the doses tested, indicating an insulin-resistant state. In parallel, insulin-stimulated GLUT4 translocation to the plasma membrane was detected in insulin sensitive (14 d) adipocytes, but this effect was significantly impaired in 21 d cells (Fig. 2B). Regarding lipid metabolism, glycerol and NEFA release was used as an index of lipolysis (Fig. 2, C and D). Maximal stimulation of lipolysis by the β-adrenergic agonist isoproterenol was observed at 14 d of differentiation (400% in glycerol release and 200% in NEFA release). However, in prolonged cultures (21 d), we observed an increase in basal glycerol and NEFA secretion and a decrease in isoproterenol responsiveness (70% in glycerol release and 150% in NEFA release). Insulin significantly inhibited isoproterenol-induced glycerol release at 14 d of differentiation, but resistance to the antilipolytic effect of insulin was detected in adipocytes differentiated for 21 d (Fig. 2, C and D).

Next, we analyzed at which molecular levels insulin sensitivity was affected. Insulin stimulation of tyrosine-phosphorylation of IRS1 and serine-phosphorylation of AKT was maximal at 14 d but resulted impaired in prolonged cultures (Fig. 2E and 2F). When examining the MAPK cascade, insulin activation of JNK1/2 and p38MAPK, but not ERK1/2, also resulted impaired in prolonged (21 d) cultures.

Human adipocyte secretion profile is regulated through differentiation; paracrine interaction between fat and skeletal muscle
When we explored the secretion pattern of LiSa-2 cells through the differentiation process, adiponectin content in conditioned medium peak at 14 d, followed by a decline on 21 d (Fig. 3A). A different pattern of release was evident with MCP-1 and IL-6, whose secretion was not detectable on 0 and 7 d differentiated cells but increased in prolonged cultures, reaching maximal levels on d 21. Because leptin and TNF-α secretion was below the detection limit in the adipocyte-conditioned media analyzed by multiplex immunoassays, mRNA expression study by QPCR was performed. Leptin mRNA accumulates in human adipocytes differentiated for 14 d but significantly decreases in prolonged cultures (Fig. 3B). By contrast, no significant differences were detected in TNF-α expression through the differentiation process (data not shown).

To test whether adipocyte secreted factors can modulate insulin sensitivity in skeletal muscle, we monitored insulin-induced glucose transport in human rhabdomyosarcoma myocytes cultured for 24 h with the different adipocyte-conditioned media. Insulin stimulation significantly increased (98%) basal glucose uptake (data not shown). Adipocyte-conditioned media from 0 and 7 d of differentiation increased insulin sensitivity on glucose uptake of 26 and 38%, respectively (Fig. 3C), and AKT phosphorylation (Fig. 3D). By con-
contrast, insulin-stimulated glucose uptake and activation of AKT was impaired in myocytes treated with 14 or 21 d adipocyte-conditioned medium, indicating the development of insulin resistance in muscle cells.

Insulin resistance in 21 d differentiated adipocytes is due to hyperinsulinemia

Furthermore, we explored whether the insulin resistance observed in adipocytes at 21 d was due to long-term treatment with insulin. For this purpose, cells were maintained with or without insulin in the differentiation medium between d 14 and 21, and glucose and lipid metabolism was analyzed. We observed a decrease in basal glucose uptake and a restoration of insulin sensitivity when adipocytes were maintained in the absence of insulin (Fig. 4A). A similar behavior was observed in insulin-induced phosphorylation of AKT (Fig. 4B). In addition, the lack of insulin also produces a decrease in basal glycerol release and a restoration on isoproterenol sensitivity at d 21 (Fig. 4, C and D). Furthermore, this mechanism seems to be dependent on perilipin A (Fig. 4E). Perilipins reside predominantly on the lipid droplets in basal conditions, but on lipolytic stimulation, these proteins are activated and move away, its detection being possible by Western blot. The lack of perilipin A activation by isoproterenol, but not on perilipin B observed at 21 d, was improved in cells cultured without insulin (Fig. 4E). In addition, a recovery on the antilipolytic effect of insulin was observed by both glycerol and NEFA release (Fig. 4, F and G).

The negative actions of long-term treatment with insulin might modulate insulin signaling through serine phosphorylation of IRS1 and/or by interfering in the expression of some phosphatases (12). As depicted in Fig. 5A, human adipocytes differentiated for 21 d showed an increase in IRS1 phosphorylation at the Ser312 residue that was not impaired when the hormone was removed in the last 7 d. However, we detected an increase in PTEN expression that was completely prevented in cells maintained without insulin. The amount of IRS1, PTP1B, PP2A, SH-PPT2, GLUT1, and GLUT4, but not FAS, remained unaltered. Nile Red staining showed hyperplasia in adipocytes differentiated for 21 d that was prevented in the absence of insulin (Fig. 5B). In this way, flow cytometry analysis showed a decrease in G0/G1 cells...
(56 vs. 65%) and a significant increase in S+G2/M cells (44 vs. 35%) in 21 d adipocytes but not in cells cultured without insulin (70% cells in G0/G1 and 30% cells in S+G2/M). These results were in agreement with an increase in cell number and a decrease in cell size (Fig. 5C).

Moreover, we explored whether the lack of insulin could restore the secretion profile. In this regard, the adiponectin release was significantly increased and the rate of MCP-1, IL-6, and NEFA secretion decreased in cells maintained without insulin (Fig. 5D). Leptin expression was also normalized in adipocytes cultured in the absence of the hormone (Fig. 5E).

Pharmacological treatment with nuclear receptors agonists ameliorates hyperinsulinemia-induced insulin resistance

To overcome insulin resistance in human adipocytes, we searched for pharmacological approaches, such as ligand activation of nuclear receptors. From the various compounds tested, only the long-term treatment with the liver X receptor (LXR) agonist, T0901317, decreased basal glucose uptake and restored insulin sensitivity (Fig. 6A), in a similar fashion as observed on AKT phosphorylation (Fig. 6B). Treatment with T0901317 increased GLUT4 and FAS protein content (Fig. 6C). Furthermore, an increase in lipid accumulation (Fig. 6D), a decrease in cell number, an enlargement in cell size (Fig. 6E), and an increase in NEFA secretion were observed in cells treated with T0901317 (Fig. 6F).

Moreover, long-term treatment with LXR agonist significantly decreased the rate of adiponectin, MCP-1, and IL-6 secretion into the medium (Fig. 6F). Finally, we also explored whether the various nuclear receptor agonists could restore insulin inhibition of lipolysis. When we analyzed glycerol (Fig. 6G) and NEFA (Fig. 6H) release, only long-term treatment with rosiglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ agonist, restored the antilipolytic effect of insulin under hyperinsulinemia conditions.
Discussion

Although glucose uptake in fat accounts for only 20% from the whole body, insulin resistance in this tissue appears to be an early and pivotal phenomenon in the development of T2D (13). We explored insulin sensitivity on glucose and lipid metabolism through human adipocyte differentiation, and an inverse biphasic response was observed: an increase on insulin sensitivity in cells differentiated for 14 d and an insulin-resistant state in prolonged cultures. Thus, long-term treatment with insulin impaired GLUT4 translocation to the plasma membrane and insulin signaling at the IRS1/AKT level. This mechanism seems to be dependent on perilipin A, an essential lipid droplet-associated protein, which functions as both a suppressor of basal lipolysis and a necessary enhancer of protein kinase A-stimulated lipolysis (16). A higher exposure of perilipin A and B in basal conditions produced by hyperinsulinemia was detected. In this regard, chronically high insulin levels inhibit β-adrenergic receptors (17), and a significant positive relationship between perilipin expression and obesity has been described (18).

An alteration of the lipolytic pathway has also been one of the major hypothesis linking insulin resistance to hyperlipidemia in obesity and T2D (15). Hyperinsulinemia induced an increase in basal lipolysis, a decrease in isoproterenol responsiveness, and impaired inhibition of lipolysis by insulin. This mechanism seems to be dependent on perilipin A, an essential lipid droplet-associated protein, which functions as both a suppressor of basal lipolysis and a necessary enhancer of protein kinase A-stimulated lipolysis (16). A higher exposure of perilipin A and B in basal conditions produced by hyperinsulinemia was detected. In this regard, chronically high insulin levels inhibit β-adrenergic receptors (17), and a significant positive relationship between perilipin expression and obesity has been described (18).

It is well documented that serine phosphorylation of IRSs impairs the normal response to insulin, and this situation has been associated with several insulin-resistant states including hyperinsulinemia (19). We detected an increase in IRS1 phosphor-
FIG. 4. Long-term treatment with insulin induces insulin resistance. Human adipocytes were maintained either in differentiation medium with insulin (normal medium) or without the hormone (−Ins) for a period of 7 d between 14 and 21 d differentiation. At the end of culture, cells were maintained overnight in serum-free and low-glucose medium. A, Cells were stimulated with 10 nM insulin (Ins) for 30 min and glucose uptake was measured. Results were expressed as percentage over basal glucose uptake of 14 d differentiated adipocytes in the absence of insulin (control 100) and are means ± se (n = 10). *, Differences between Ins vs. its absence; ○, differences vs. basal values of 14 d differentiation cells; △, differences vs. basal values of 21 d differentiation cells. B, Cells were cultured as indicated above and stimulated or not with 10 nM Ins for 10 min. Cell lysates were analyzed by Western blot with the corresponding antibodies against phosphorylated and total AKT. The autoradiograms were quantified by scanning densitometry, and the results shown are representative of at least four independent experiments. *, Differences between Ins vs. its absence; ○, differences vs. values of 14 d differentiation cells; △, differences vs. values of 21 d differentiation cells. C, Cells were stimulated with 1 μM isoproterenol (Isoprot) for 15 min, and glycerol release was measured. Results are means ± se (n = 10) and were expressed as percentage over lipolytic rate of 14 d differentiated adipocytes in the absence of Isoprot (control 100). Statistical significance was established as in A. D, Data on glycerol release were also expressed as the percentage of stimulation over basal in the absence of Isoprot. Statistical significance as in B. E, Cell lysates were analyzed by Western blot with the corresponding antibodies against HSL, perilipin A (PerA), and perilipin B (PerB). The results shown are representative of at least three independent experiments. F, Adipocytes were stimulated with several doses of Ins for 1 h, prior to isoproterenol stimulation, and glycerol release was measured. Results were expressed as percentage over isoproterenol-induced lipolysis (control 100) and are means ± se (n = 10). *, Differences between Isoprot + Ins vs. Isoprot. G, Human adipocytes were stimulated with 10 nM Ins prior to Isoprot stimulation, and NEFA release was measured. Results were expressed as percentage over basal values (100). *, Differences between Isoprot vs. its absence; △, differences between Isoprot + Ins vs. Isoprot.
ylation at the Ser312 residue and a decrease in insulin-induced IRS1 tyrosine phosphorylation, without changes in IRS1 expression. This mechanism differs from the decrease in IRS1 levels described in 3T3-L1 adipocytes (20). However, our results demonstrate that hyperinsulinemia itself is unlikely to account for the increase in IRS1 serine phosphorylation because it was not impaired when human adipocytes were in the absence of insulin.

Furthermore, we found an increase in PTEN expression in hyperinsulinemia impairs insulin signaling and deregulates secretion pattern on human adipocytes. Human adipocytes were cultured in the differentiation medium with or without insulin as indicated above. A, Cell lysates were also analyzed by Western blot with antiphospho-IRS1 (Ser312), IRS1, PTP1B, PP2A, PTEN, SH-PTP2, GLUT1, GLUT4, and FAS. The autoradiograms were quantified by scanning densitometry, and the results shown are representative of at least four independent experiments. B, Nile Red staining was performed after the treatments. Representative experiments of three are shown. C, Cell number (white bars) and cell size (black bars) analyzed by flow cytometry were determined. D, LiSa-2 cells were cultured overnight in serum-free medium before to obtain the different adipocyte-conditioned medium. Next, adiponectin, MCP-1, IL-6, and NEFA release were analyzed. E, Leptin expression was analyzed by qPCR. Data were expressed as percentage over undifferentiated cells (100). *, Differences vs. values at 14 d cells; △, differences vs. values at 21 d cells. Results in C and D are means ± SE (n = 4).
FIG. 6. Nuclear receptor agonists ameliorate hyperinsulinemia-induced insulin resistance. A, Human adipocytes were maintained in normal differentiation medium in the absence or presence of different nuclear receptor agonists. The ligands used were 10 μM rosiglitazone (Rosi) as PPARγ agonist, 3 μM T0901317 (T1317) as LXR agonist, 10 μM TTNPB as retinoid acid receptor agonist, or 20 μM phytanic acid (PA) as retinoid X receptor agonist for a period of 7 d between 14 and 21 d differentiation. At the end of culture, cells were maintained overnight in serum-free and low glucose medium prior stimulation with 10 nM insulin (Ins) for 30 min and glucose uptake was measured. Results were expressed as percentage over basal glucose uptake of 14 d adipocytes (control × 100) and are means ± SE (n = 10). *, Differences between Ins vs. its absence; ○, differences vs. basal values of 14 d cells; △, differences vs. basal values of 21 d cells. B, Adipocytes treated with T1317 as described above were stimulated with or without 10 nM Ins for 10 min. Cells were lysed and total protein was submitted to Western blot and immunodetected with anti-P-AKT and total AKT. The autoradiograms were quantified by scanning densitometry, and the results shown are representative of at least four independent experiments. *, Differences between Ins vs. its absence; △, differences vs. values of 14 d cells; ●, differences vs. values of 21 d cells. C, Cell lysates were also analyzed by Western blot with anti-GLUT4, GLUT1, and FAS. The autoradiograms were quantified by scanning densitometry, and the results shown are representative of at least four independent experiments. Statistical significance was tested as in B. D, Phase-contrast and Nile Red staining images (magnification, ×40) of the cells were taken after the treatments. E, Cell number (white bars) and cell size (black bars) analyzed by flow cytometry were determined. Statistical significance was
21 d adipocytes. This effect was completely resolved when insulin was removed from the medium, allowing AKT phosphorylation. It is worth mentioning that hyperinsulinemia was not produced in muscle-specific PTEN-deficient mice (21), whereas inhibition of PTEN expression in ob/ob mice reduced insulin concentrations (22).

We also explored the endocrine function of adipose tissue and observed that human adipocytes release different adipokines through the differentiation process. Thus, adiponectin secretion was in keeping with insulin sensitivity through adipogenesis, in agreement with its inverse correlation described to insulin-resistant states (23). By contrast, secretion of proinflammatory cytokines, such as MCP-1 and IL-6, as well as NEFA release, were markedly stimulated in human adipocytes differentiated for 21 d. In this regard, elevated plasma concentrations of MCP-1, IL-6, and NEFA have been detected in obese and diabetic patients (24–26). Furthermore, this study reported that hyperinsulinemia might account for the decrease of adiponectin release and leptin expression and the increase of MCP-1, IL-6, and NEFA secretion. Insulin was found to suppress mRNA expression of adiponectin receptors in hepatocytes (27) and increase MCP-1 expression in murine adipocytes (28). A similar behavior to adiponectin secretion was also observed with leptin expression. Although this adipokine is positively regulated by insulin in humans and rodents (29), down-regulation in prolonged cultures might be consequence of increased NEFA release, as previously described in murine adipocytes (30).

In an attempt to study the potential crosstalk between human fat and skeletal muscle, we analyzed the effect of adipocyte-conditioned media on insulin sensitivity. As in human adipocytes, we also detected an inverse biphasic response in myocytes. Thus, signals coming from undifferentiated adipocytes or those differentiated for 7 d enhanced insulin-induced glucose uptake and AKT phosphorylation, whereas signals from more differentiated adipocytes induced insulin resistance. These results appear to represent a paradox because adipocytes undifferentiated or poorly differentiated induce insulin resistance in skeletal muscle, despite the fact that they do not respond to insulin. However, we cannot rule out that lower adiponectin secretion at these stages, or the presence of other adipokines, could be enough to increase insulin sensitivity in skeletal muscle. By contrast, conditioned medium from 14 or 21 d adipocytes induced insulin resistance on glucose uptake in human myocytes, despite the higher concentration of adiponectin. Nevertheless, MCP-1, IL-6, and NEFA release at these stages were markedly stimulated. In this regard, an incapacity of skeletal muscle of obese and diabetic individuals to respond to adiponectin has been recently reported (31). On the other hand, MCP-1 impairs insulin signaling and glucose uptake in skeletal muscle at concentrations lower even than those found in the circulation (32). Prolonged exposure to NEFA inhibits the insulin signaling pathway (33), and although the role of IL-6 in the etiology of insulin resistance is not fully understood, pretreatment with this cytokine in vivo reduced insulin-stimulated glucose uptake (34).

To restore insulin sensitivity in insulin-resistant human adipocytes, we used a pharmacological approach by testing several agonists of nuclear receptors. We observed that the synthetic LXR agonist T0901317 restored insulin-stimulated glucose uptake, in parallel to the recovery of insulin-induced AKT phosphorylation, in agreement with the restoration found in TNF-α-treated brown adipocytes (9). Moreover, treatment of human adipocytes with T0901317 induced an increase in GLUT4 and FAS expression. GLUT4 promoter is a direct transcriptional target for the LXR/retinoid X receptor heterodimer (35), but the role of LXR in adipogenesis is a matter of controversy (36, 37). In addition, our studies demonstrate for the first time that LXR activation has beneficial effects on the secretion pattern because it inhibits MCP-1 and IL-6 secretion in insulin-resistant human adipocytes, in a similar fashion as previously reported in astrocytes (38).

The lack of effect of the PPARγ agonist rosiglitazone on recovery of glucose uptake might be surprising. Nevertheless, an increase in basal glucose uptake was detected, in agreement with the results obtained in obese murine models (39). Furthermore, rosiglitazone completely restored the antilipolytic effect of insulin under hyperinsulinemia conditions. The improvement in lipid metabolism in insulin-resistant human adipocytes agrees with metabolic actions of rosiglitazone in vivo such as decreasing postprandial NEFA concentrations in T2D (40).

In conclusion, hyperinsulinemia induced a deregulation of adipokines and NEFA secretion by human adipocytes that produced insulin resistance on adipocytes as well as on myocytes, in which this insulin-resistant state is detected earlier. Moreover, this work supports the hypothesis of nuclear receptor as interesting targets for drug treatment of insulin resistance. Thus, LXR agonists improve insulin sensitivity on glucose metabolism, whereas PPARγ agonist shows beneficial effects on lipid metabolism.

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This work was supported by Grants BFU-2005-03034 from Ministerio de Educacion y Ciencia; S-SAL-0159-2006 from Comunidad de Madrid; PR34/07-15887 from Santander/Complutense University; and tested as in B. F. Adiponectin, MCP-1, IL-6, and NEFA release were analyzed in the different adipocyte-conditioned media. Data were expressed as picograms per milliliter for adipokines and micromoles for NEFA. Statistical significance was tested as in B. G. Human adipocytes were cultured in the picograms per milliliter for adipokines and micromoles for NEFA. Statistical significance was tested as in B. G. Human adipocytes were cultured in the medium from 14 or 21 d adipocytes induced insulin resistance on glucose uptake, allowing AKT phosphorylation. Nevertheless, MCP-1, IL-6, and NEFA release were markedly stimulated in human adipocytes differentiated for 21 d. In this regard, elevated plasma concentrations of MCP-1, IL-6, and NEFA have been detected in obese and diabetic patients (24–26). Furthermore, this study reported that hyperinsulinemia might account for the decrease of adiponectin release and leptin expression and the increase of MCP-1, IL-6, and NEFA secretion. Insulin was found to suppress mRNA expression of adiponectin receptors in hepatocytes (27) and increase MCP-1 expression in murine adipocytes (28). A similar behavior to adiponectin secretion was also observed with leptin expression. Although this adipokine is positively regulated by insulin in humans and rodents (29), down-regulation in prolonged cultures might be consequence of increased NEFA release, as previously described in murine adipocytes (30).

In an attempt to study the potential cross talk between human fat and skeletal muscle, we analyzed the effect of adipocyte-conditioned media on insulin sensitivity. As in human adipocytes, we also detected an inverse biphasic response in myocytes. Thus, signals coming from undifferentiated adipocytes or those differentiated for 7 d enhanced insulin-induced glucose uptake and AKT phosphorylation, whereas signals from more differentiated adipocytes induced insulin resistance. These results appear to represent a paradox because adipocytes undifferentiated or poorly differentiated induce insulin resistance in skeletal muscle, despite the fact that they do not respond to insulin. However, we cannot rule out that lower adiponectin secretion at these stages, or the presence of other adipokines, could be enough to increase insulin sensitivity in skeletal muscle. By contrast, conditioned medium from 14 or 21 d adipocytes induced insulin resistance on glucose uptake in human myocytes, despite the higher concentration of adiponectin. Nevertheless, MCP-1, IL-6, and NEFA release at these stages were markedly stimulated. In this regard, an incapacity of skeletal muscle of obese and diabetic individuals to respond to adiponectin has been recently reported (31). On the other hand, MCP-1 impairs insulin signaling and glucose uptake in skeletal muscle at concentrations lower even than those found in the circulation (32). Prolonged exposure to NEFA inhibits the insulin signaling pathway (33), and although the role of IL-6 in the etiology of insulin resistance is not fully understood, pretreatment with this cytokine in vivo reduced insulin-stimulated glucose uptake (34).

To restore insulin sensitivity in insulin-resistant human adipocytes, we used a pharmacological approach by testing several agonists of nuclear receptors. We observed that the synthetic LXR agonist T0901317 restored insulin-stimulated glucose uptake, in parallel to the recovery of insulin-induced AKT phosphorylation, in agreement with the restoration found in TNF-α-treated brown adipocytes (9). Moreover, treatment of human adipocytes with T0901317 induced an increase in GLUT4 and FAS expression. GLUT4 promoter is a direct transcriptional target for the LXR/retinoid X receptor heterodimer (35), but the role of LXR in adipogenesis is a matter of controversy (36, 37). In addition, our studies demonstrate for the first time that LXR activation has beneficial effects on the secretion pattern because it inhibits MCP-1 and IL-6 secretion in insulin-resistant human adipocytes, in a similar fashion as previously reported in astrocytes (38).

The lack of effect of the PPARγ agonist rosiglitazone on recovery of glucose uptake might be surprising. Nevertheless, an increase in basal glucose uptake was detected, in agreement with the results obtained in obese murine models (39). Furthermore, rosiglitazone completely restored the antilipolytic effect of insulin under hyperinsulinemia conditions. The improvement in lipid metabolism in insulin-resistant human adipocytes agrees with metabolic actions of rosiglitazone in vivo such as decreasing postprandial NEFA concentrations in T2D (40).

In conclusion, hyperinsulinemia induced a deregulation of adipokines and NEFA secretion by human adipocytes that produced insulin resistance on adipocytes as well as on myocytes, in which this insulin-resistant state is detected earlier. Moreover, this work supports the hypothesis of nuclear receptor as interesting targets for drug treatment of insulin resistance. Thus, LXR agonists improve insulin sensitivity on glucose metabolism, whereas PPARγ agonist shows beneficial effects on lipid metabolism.
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