Insulin Sensitization of Human Preadipocytes through Glucocorticoid Hormone Induction of Forkhead Transcription Factors


Glucocorticoids promote adipogenesis in vivo and when present in excess promote the development of visceral obesity and insulin resistance, hallmarks of the metabolic syndrome (1, 2). Thus, patients with Cushing’s syndrome, in which adrenal glucocorticoid production is elevated subsequent to a pituitary adenoma, develop truncal obesity and manifest a pathophysiology similar to the metabolic syndrome (3). Similarly, the development of truncal obesity is a side effect of exogenous glucocorticoid therapy (4).

Recently, it has been recognized that glucocorticoids are also produced locally in a number of tissues, including in mature white adipocytes, through the action of 11β-hydroxysteroid dehydrogenase 1 (11βHSD1). There is a growing recognition from rodent and human correlative studies as well as in murine genetic models that 11βHSD1 activity plays a key role in the development of the metabolic syndrome and visceral obesity (5, 6). In particular, local synthesis is likely to play a role in the contribution of glucocorticoids to insulin resistance in adipocytes (7–9).

Insulin promotes the energy storage function of white adipose tissue (WAT) in response to caloric excess by inducing glucose uptake by mature adipocytes and enhancing lipogenesis while inhibiting lipolysis (10). In addition, insulin promotes the differentiation of preadipocytes to increase adipose tissue storage capacity. Insulin acts through the insulin receptor (IR), a tyrosine kinase receptor that mediates tyrosine phosphorylation of IR

**Glucocorticoids are synthesized locally in adipose tissue and contribute to metabolic disease through the facilitation of adipose tissue expansion. Here we report that exposure of human primary preadipocytes to glucocorticoids increases their sensitivity to insulin and enhances their subsequent response to stimuli that promote differentiation. This effect was observed in primary human preadipocytes but not in immortalized 3T3-L1 murine preadipocytes or in fully differentiated primary human adipocytes. Stimulation of insulin signaling was mediated through induction of insulin receptor (IR), IR substrate protein 1 (IRS1), IRS2, and the p85 regulatory subunit of phosphoinositide-3-kinase, which led to enhanced insulin-mediated activation of Akt. Although induction of IRS2 was direct, induction of IR and IRS1 by glucocorticoids occurred subsequent to primary induction of the forkhead family transcription factors FoxO1A and FoxO3A. These results reveal a new role for glucocorticoids in preparing preadipocytes for differentiation.** (Molecular Endocrinology 24: 104–113, 2010)
substrates (IRS) IRS1 and IRS2, which direct the activation of adipogenic signaling pathways (11). Genetic and cellular approaches have demonstrated that insulin is required for adipogenesis (10, 12) and mediates its effects predominantly through activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway (13–18).

In preadipocyte cell culture models, the addition of glucocorticoids to the culture medium together with adipogenic stimuli potentiates preadipocyte differentiation. This enhancement of differentiation is mediated primarily through the induction and potentiation of the transcriptional activity of CCAAT enhancer-binding protein (C/EBP) family members that initiate the transcriptional cascade that mediates differentiation (19–23).

By contrast to cell culture models of adipocyte differentiation where glucocorticoids are added at the beginning of differentiation, the local 11βHSD1 activity present in WAT in vivo provides for the continuous exposure of preadipocytes to glucocorticoid. Here we have determined that exposure of primary human WAT preadipocytes to synthetic glucocorticoid dexamethasone (dex) exhibits a priming effect that strongly enhances subsequent differentiation without replacing the later effects of steroid in the differentiation cocktail.

Dex treatment of naive preadipocytes up-regulated key components of the insulin signaling pathway, including IR, IRS1, IRS2, and the p85α PI3K regulatory subunit, which led to the enhancement of protein kinase B (Akt) activation in response to insulin when differentiation was stimulated. These effects were specific to primary human preadipocytes, with dex treatment failing to enhance insulin signaling in primary cultures of differentiated adipocytes or in immortalized murine preadipocytes.

Dissection of the steroid signaling pathway in the primary preadipocytes indicated that induction of IR and IRS1 occurred over 24–48 h and depended on the prior induction the forkhead transcription factors forkhead box O1A (FoxO1A) and FoxO3A, whereas IRS2 was rapidly induced in a manner consistent with reports showing it to be a direct target for the glucocorticoid receptor (GR) in other tissues. These results identify a new pathway through which the adipogenic effects of glucocorticoids are mediated and emphasize the differential sensitivity of preadipocytes and adipocytes to steroid.

Results

Glucocorticoids prime human primary preadipocytes for differentiation through enhancement of insulin signaling

To assess the effect of exposure of preadipocytes to glucocorticoids before the onset of differentiation, we pretreated confluent primary human preadipocytes with 10−6 M dex for 48 h before the stimulation of differentiation. This pretreatment enhanced subsequent differentiation of the preadipocytes as reflected by Oil Red O staining of neutral lipid content (upper) and Western analysis of aP2 protein expression (lower) from human adipocytes differentiated for 14 d. Differentiation was preceded by 48 h culture of the cells with vehicle (left) or 1 μM dex (right, +dex). To initiate differentiation, cells were treated with MIX and insulin (MI) or MIX, insulin, and 1 μM dex (MID).

FIG. 1. Glucocorticoid treatment of naive preadipocytes enhances subsequent differentiation and induces components of the insulin signaling pathway. A, Photomicrographs of Oil Red O staining of neutral lipids content (upper) and Western analysis of aP2 protein expression (lower) from human adipocytes differentiated for 14 d. Differentiation was preceded by 48 h culture of the cells with vehicle (left) or 1 μM dex (right, +dex). To initiate differentiation, cells were treated with MIX and insulin (MI) or MIX, insulin, and 1 μM dex (MID). Scale bar, 1 mm. Results are representative of a minimum of five repeats. B, Microarray analysis derived AFC of insulin signaling components in human preadipocytes. AFC of mRNA expression compared with control cells was determined using RMA analysis, and statistical validation was calculated using FDR-CI analysis. C, qRT-PCR-derived average fold induction in mRNA abundance at d0 of IR, IRS1, IRS2, and PI3KR1 in dex-pretreated (+) compared with control (−) human preadipocytes. For analysis, n = 3, each performed in duplicate. Data are plotted ±SEM. *P ≤ 0.05, **P ≤ 0.01 as determined using a Student’s paired t test. D, Western analysis of protein expression at d 0 in control (−) and dex-pretreated (+) cells. Average fold induction ±SD was calculated by densitometry using ImageQuant software (n ≥ 3). P value was determined as described in C: †, P ≤ 0.1; *, P ≤ 0.05; **, P ≤ 0.01; ***P ≤ 0.001. Hs., Homo sapiens.
analysis followed by false discovery rate (FDR)-confidence interval (CI)-based statistical validation (24, 25). In these experiments, C/EBPβ, an established transcriptional target of GR was induced 1.6-fold. Therefore, to establish a reasonable expectation of capturing a cohort of glucocorticoid-responsive genes, we used a minimal average fold change (AFC) of 1.5 for our analysis. By these criteria, we identified approximately 550 genes whose expression was altered at least 1.5-fold by glucocorticoids as compared with control cells.

Interestingly and somewhat unexpectedly, analysis of the microarray data showed 1.6- to 3.8-fold enrichment in the mRNAs for key components of the insulin signaling pathway, IR, IRS1, IRS2, and PI3K regulatory subunit 1 (PI3KR1), when preadipocytes were treated with dex (Fig. 1B). Quantitative RT-PCR (qRT-PCR) analysis confirmed the significance of the induction, with the mRNA induction of these factors ranging from 2.5- to 6-fold after dex treatment (Fig. 1C). A concomitant and proportional increase in the levels of each protein was observed (Fig. 1D).

Activation of the signaling cascade downstream of the IR is rapid with maximal IRS1 and Akt activation occurring within the first 5 min (26, 27). IRS signaling declines rapidly from its peak, whereas Akt activation persists. To determine whether the up-regulation of insulin signaling pathway components by dex translated into increased response to insulin in the preadipocytes, we evaluated total cellular tyrosine phosphorylation and activation of Akt after 5 min and 1 h stimulation with 100 nM insulin (Fig. 2). In the first instance, dex-treated cells displayed strongly enhanced tyrosine phosphorylation [phospho- (P-)Tyr] after 5 min of insulin treatment (Fig. 2A). In particular, there was an enhanced induction of the P-Tyr signal that overlaid with the migration of IRS1 and IRS2 (~185 kDa) (Fig. 2A and data not shown). The increase in P-Tyr translated further into increased activation of the downstream effector Akt, as visualized by the levels of Akt phosphorylation at Ser473. Although phosphorylated tyrosine induction was transient, being almost completely attenuated by 1 h, Akt remained readily detectable at 1 h in the preadipocytes that had been exposed to glucocorticoids (Fig. 2B), representing a significant increase in downstream insulin signaling.

Titration of the concentration of glucocorticoid required to enhance insulin signaling showed that 10^{-9} M dex pretreatment resulted in a modest enhancement in the induction of both P-Akt and P-Tyr after 5 min of insulin treatment (P-Akt, P = 0.08), with this induction reaching strong statistical significance (P-Akt, P ≤ 0.05) by 10^{-8} M dex (Fig. 2C). Thus, dex enhancement fell within the range expected for the physiological relevance of a glucocorticoid effect (28).

Our results contrast with reports that glucocorticoids antagonize insulin signaling in primary human omental adipocytes and adipocytes differentiated from the immortalized murine 3T3-L1 and 3T3 F442A preadipocyte cell lines (7–9). Indeed, enhancement of insulin sensitivity by dex may be a specific property of the primary preadipocyte, because dex pretreatment of immortalized 3T3-L1 preadipocytes had no significant effect on the levels of IR, IRS1, or IRS2 and repressed the Akt response to insulin (Fig. 3A, i–iii and v). By contrast, C/EBPβ, a known direct transcriptional target of glucocorticoids in 3T3-L1 preadipocytes was induced 3.4 ± 0.2-fold under the same conditions, confirming that these cells were able to respond to glucocorticoids (Fig. 3A, iv). Furthermore, induction of IR and IRS1 exhibited a similar resistance to
glucocorticoid treatment in primary human adipocytes that had been differentiated in culture under optimal adipogenic conditions for 12 d (29) and withdrawn from glucocorticoids for 48 h before restimulation (Fig. 3B). IRS1 induction was completely abrogated, whereas IR induction was markedly reduced under these conditions. By contrast, the fold IRS2 response to glucocorticoids was maintained in the mature adipocytes. Thus, the profile of glucocorticoid responsiveness of insulin signaling components varied between preadipocytes and mature adipocytes and in an established murine preadipocyte cell line.

**Glucocorticoids act through FoxO1A and FoxO3A to induce IR and IRS1 in primary human preadipocytes**

Time-course analysis of IR, IRS1, and IRS2 induction in human preadipocytes revealed a rapid 3-fold induction of IRS2 mRNA within 4 h of dex treatment of primary human preadipocytes, whereas the induction of IR and IRS1 were delayed for at least 24 h (Fig. 4). The rapid IRS2 response was consistent with the previous report of glucocorticoid response elements (GREs) that mediated glucocorticoid- and progesterin-dependent induction of IRS2 in HeLa cells and glucocorticoid-dependent induction of a human IRS2 promoter-driven luciferase construct in rat primary hepatocytes (30, 31). By contrast, the delayed response of IR/IRS1 suggested an intermediate step in their induction.

Further analysis of our microarray data suggested FoxO1A and FoxO3A as potential glucocorticoid-dependent intermediary factors for the induction of IR and IRS1 (Fig. 5A). Both factors had previously been shown through chromatin immunoprecipitation (ChIP) and transcription assays to directly induce transcription of IR in a manner that was conserved from mammals to *Drosophila* (30, 32, 33), whereas the IRS1 gene regulatory region also contains a series of elements that match closely with known FoxO transcriptional regulatory motifs (supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org).
The qRT-PCR analysis set FOXO1A mRNA induction after glucocorticoid treatment at 7.9 ± 1.0-fold and FOXO3A mRNA at 3.1 ± 0.7-fold (Fig. 5B). For both FOXO1A and FOXO3A, mRNA induction occurred within 4 h, and their mRNA levels remained elevated for the duration of the steroid treatment (Fig. 5C). A previous report has shown that a mutation that compromises GR dimerization and DNA binding activity alleviates glucocorticoid-mediated induction of FoxO1A (34), suggesting a possible direct regulation. Consistent with this observation, cycloheximide treatment of the preadipocytes failed to attenuate the induction of FoxO1A, FoxO3A (Fig. 5D), or IRS2 mRNA (supplemental Fig. 2). ChIP analysis using a primer set flanking GRE motifs upstream of the FoxO1A promoter verified recruitment of GR to the region between 1.1 and 1.4 kb upstream of the promoter within 30 min of dex treatment (Fig. 5E).

Induction of FoxO1A and FoxO3A mRNA was also observed at a reduced level in mature primary human adipocytes (Fig. 5F). By contrast, in 3T3-L1 preadipocytes, FoxO1A and FoxO3A responded minimally to dex treatment, with only FoxO1A mRNA modestly induced (1.9 ± 0.2-fold) (Fig. 5G).

To directly test the role of FoxO1A and FoxO3A in the induction of IR and IRS1 by glucocorticoids in the primary human preadipocyte, we ectopically expressed a dominant-negative construct of FoxO1A (F1DN) that would interfere with both FoxO1A and FoxO3A transcriptional regulation (35). F1DN, which contains an intact FoxO1A DNA-binding domain but lacks a C-terminal transactivation domain was abundantly expressed from a lentiviral expression vector and was localized to the nucleus of the primary human preadipocytes (supplemental Fig. 3). Expression of F1DN, but not control virus, prevented the dex-mediated induction of IR and IRS1 (Fig. 6,A and B). It similarly strongly reduced the dex-dependent enhancement of IRS1 and Akt phosphorylation in response to insulin (Fig. 6C). The small dex-dependent enhancement of IRS1 and Akt induction that remained correlated with the inability of F1DN to reduce the induction of IRS2 by dex (Fig. 6A). Together, these results provide compelling evidence that glucocorticoid exposure of preadipocytes enhances their subsequent differentiation through mechanisms that include increasing insulin sensitivity of the preadipocyte through the induction of IR, IRS1, and IRS2.

**Discussion**

Glucocorticoids promote adipogenesis. In this work, we have identified a novel means through which this is accomplished, by enhancing the sensitivity of naive preadipocytes to insulin, one of the signals that directly activate differentiation. This response may be human specific because it does not occur in the murine 3T3-L1
preadipocyte model or may reflect a property of primary preadipocytes that is lost from immortalized cell lines. Enhancement of insulin sensitivity by glucocorticoids appeared to be mainly indirect and dependent on the initial induction of FoxO1A and FoxO3A, which have been previously established as direct regulators of IR and which we show here to also regulate IRS1 expression (32, 33). Thus, it would seem that local production of glucocorticoids within the adipose tissue through 11BHS2 activity provides an environment in WAT that prepares preadipocytes for differentiation in response to metabolic cues such as elevated insulin levels in response to caloric excess.

An important feature of our results was that glucocorticoid pretreatment enhanced subsequent differentiation of the preadipocytes but did not blunt the additional stimulatory effects of glucocorticoids included in the adipogenic cocktail that drives differentiation. This is consistent with the early effects of glucocorticoids on insulin sensitivity being followed, upon the initiation of differentiation, by the well characterized additional effects of GR on Pref-1, C/EBPβ, C/EBPδ, and other factors in the regulatory cascade that directs preadipocyte differentiation, such as the transcriptional coregulators including histone deacetylase 1 (HDAC1) and general control of amino acid synthesis 5-like 2 (GCN5) (10, 20–22, 36, 37). Glucocorticoid pretreatment also led to the early induction of C/EBPδ, a direct target of GR that contributes to subsequent differentiation. However, the glucocorticoids coincidentally induced the dominant-negative C/EBP, CHOP, to a similar level and the subsequent profile of C/EBPδ expression from the onset of differentiation was unchanged in the human preadipocytes by dex pretreatment (data not shown). CHOP has been shown to suppress the transcriptional effects of C/EBPδ and C/EBPβ until it is down-regulated 12–24 h after the stimulation of differentiation (38). Furthermore, C/EBPβ also was up-regulated by dex in 3T3-L1 preadipocytes without affecting insulin sensitivity. Thus, the up-regulation of C/EBPβ was insufficient for the increase in insulin sensitivity. It also seems unlikely to account for the effects of dex on subsequent differentiation.

Sensitization of the insulin signaling pathway by glucocorticoid treatment was linked to the up-regulation of the forkhead transcription factors FoxO1A and FoxO3A, which in turn promoted the expression of IR and IRS1 in the dex-treated preadipocytes. Proteins from the FoxO family serve as master switches for key cellular processes including proliferation and cell cycle, cell survival and apoptosis, differentiation, and oxidative stress resistance (39). FoxO proteins have previously been shown to be required for both preadipocyte differentiation and adipose tissue function (40–42).

Although previous reports have firmly established FoxO transcription factors as direct transcriptional regulators of the IR gene through sequence-specific binding to a forkhead response element in its promoter region in human 293 cells, murine C2C12 cells as well as in Drosophila and Drosophila-derived cell lines (32, 33), we provide the first indication that the FoxOs are also transcriptional regulators of IRS1 expression. The coincident induction of IR and IRS1, together with predicted forkhead-response elements in the promoter region of the IRS1 gene (supplemental Fig. 1) suggest that IRS1 is also a direct FoxO target. Confirmation of IRS1 regulation awaits detailed transcriptional regulatory studies including the development of ChIP in the primary preadipocytes.

FoxO transcription factors are regulated both transcriptionally and through posttranslational modifications that control their subcellular localization (43). Thus, the delay between induction of FoxO1A/3A and IR/IRS1 in response to dex may reflect subsequent events required for FoxO1A/3A to require transcriptional competency. Alternatively, it may be dependent on the induction or
down-regulation of other factors that interact functionally with FoxO1A/3A. Regardless of this, however, within the context of the preadipocytes in vivo, where dex exposure is constant, this delay is unlikely to be of significance to IR/IRS1 levels.

Our studies show that IRS2, FoxO1A, and FoxO3A are downstream targets of glucocorticoid action in the preadipocyte. Here we have shown that that FoxO1A is a direct target of GR (Fig. 5E). Further studies are required to determine whether putative GREs identified in the promoter regions of FoxO3A and IRS2 are physically recognized by GR. However, dex-dependent induction of all three genes was observed within 4–6 h and was not blocked by cycloheximide, an inhibitor of protein synthesis (Figs. 5D and supplemental Fig. 2) that would be expected to abrogate activation if the effect of steroid required expression of an intermediary factor. Furthermore, our results are consistent with a very recent report that FoxO1A and FoxO3A expression are induced by dex in mice, and this induction is reduced in GRdim/dim mice in which GR binding to its transcriptional regulatory elements is compromised (34).

The mechanisms that regulate FoxO involvement in adipogenesis during differentiation have been studied in detail and involve both transcriptional and posttranscriptional control. Notably, activation of the insulin signaling pathway at the onset of differentiation leads to the functional inactivation of FoxO1A through its exclusion from the nucleus subsequent to phosphorylation by Akt (43). This is enhanced by sirtuin 2-mediated acetylation of FoxO1A (40). By d 4 of differentiation, FoxO1A phosphorylation is lost, and it regains its transcriptional regulatory potential as it returns to the nucleus (43).

Our results identify FoxO1A and FoxO3A as transcriptional regulatory targets of glucocorticoids and IRS1 as a regulatory target of the FoxO proteins that act to enhance the insulin sensitivity of preadipocytes. As summarized in Fig. 7, exposure of preadipocytes to glucocorticoids increases FoxO1A/FoxO3A gene expression and the levels of transcriptionally active protein and drives IRS2 expression. The elevation in FoxO1A/FoxO3A in turn leads to the up-regulation of IR and IRS1 expression to prime the preadipocytes for differentiation by increasing their sensitivity to insulin. At the onset of differentiation, insulin-mediated activation of Akt feeds back to down-regulate insulin sensitivity by inactivating at least FoxO1A. The enhancement of Akt signaling by glucocorticoids would render the subsequent inactivation of FoxO1A more efficient. Thus, although our results show that expression of a dominant-negative FoxO1A reduces initial sensitivity of the cells to insulin, they are also consistent with a dominant-negative enhancement of the subsequent downstream inactivation of FoxO1A by Akt, leading to a subsequent increase in differentiation (42).

To further explore the linkage of FoxO1A to the glucocorticoid-mediated induction of IR and IRS1, we also attempted to express full-length constitutively active FoxO1A and FoxO3A constructs. However, several versions of these constructs expressed only poorly in the primary preadipocytes (data not shown) and could not be assessed for effects on insulin signaling.

Significantly, the glucocorticoid-mediated sensitization of insulin signaling was not observed in the murine 3T3-L1 preadipocyte model. This difference correlated with the resistance of FoxO1A and FoxO3A induction by dex in these cells. Because C/EBPβ was still induced by dex and FoxO1A and FoxO3A are expressed in these cells (Fig. 3A, iv), our results suggest a specific defect or imprint in the cells that prevent the GR from stimulating FoxO1A/FoxO3A transcription. This presumptive defect highlights the importance and advantages of studying preadipocyte differentiation in cells that provide the closest possible model for the native human preadipocyte.

Differential regulation of insulin sensitivity in preadipocytes and adipocytes by glucocorticoids expands the mechanisms through which glucocorticoids appear to support the development and maintenance of healthy WAT. Adipose tissue responds to caloric excess by either increasing adipocyte number through the recruitment and differentiation of preadipocytes (hyperplasia) and/or increasing their lipid accumulation (hypertrophy). Thus, it tempting to speculate that local synthesis of glucocorticoids through 11βHSD1 reflects an attempt by WAT to alleviate hypertrophy through promoting the production of new adipocytes.
Materials and Methods

Plasmids and reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The pCMX-Flag-humFoxO1A DN plasmid was a kind gift from Dr. Accili and has been described previously (35). The construct was subcloned into pcDH1-MCS1-EF1-puro (Systems Biosciences, Mountain View, CA) using Swal and BamHI restriction sites.

Cell maintenance and differentiation

Cryopreserved, sc human primary preadipocytes from female donors with a normal body mass index (22.5 ± 0.2 kg/m²) were purchased from Zen-Bio Inc. (Research Triangle Park, NC). Cells were maintained at 5% CO₂ in DMEM with 1.0 g/liter glucose, 20% calf serum (Life Technologies, Inc., Burlington, Canada), 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 U/ml nystatin. Cells were seeded into Nunc T75 flasks and expanded once before differentiating. Medium was replaced every 2 d. Murine 3T3-L1 preadipocytes were maintained at 10% CO₂ in DMEM with 1.0 g/liter glucose and 10% calf serum.

For exposure of preadipocytes to glucocorticoids before differentiation, both human and 3T3-L1 preadipocytes were seeded into Nunc 12-well culture dishes. Upon reaching confluence, preadipocytes were stimulated with 1 μM dexamethasone or vehicle for 1 h. Cells were then washed once with PBS, stained with 2 ml of Oil Red O mixture, and washed twice with PBS and fixed with 2 ml 10% formalin in PBS. To assess neutral lipid content, mature adipocytes were treated with 100 nM insulin, 0.5 mM 3-isobutyl-1-methylxantine and 50 U/ml nystatin. To stimulate differentiation, the cells were maintained and treated with dex upon reaching confluence exactly as described above. After 48 h stimulation with 1 μM dex (d 0), total RNA was harvested using the QIAGEN (Valencia, CA) RNeasy kit. RNA from three wells was pooled for subsequent microarray analysis. Additionally, RNA from three wells was pooled and stored at −80 °C for future RT-PCR-based validation of microarray results.

Real-time PCR analysis of mRNA expression

The qRT-PCR was performed as previously described (20). GAPDH was used as an internal control for all experiments (45), with the exception of the experiments in Fig. 6, in which β-actin was used. The primer pairs used for each target are listed in the supplemental Table 1. Data represent relative mRNA abundance from a minimum of three experiments each done in duplicate. Data are shown ±SEM. Statistical significance was determined using two-tailed, paired Student’s t tests.

To measure mRNA abundance after glucocorticoid stimulation in the presence of cycloheximide, confluent human primary preadipocytes were stimulated with vehicle, 1 μM dex, and 2 μg/ml cycloheximide as indicated for 8 h. Cells treated with cycloheximide were pulsed with 2 μg/ml cycloheximide for 15 min before subsequent stimulation.

ChIP assay

Potential GREs were identified between 1.1 and 1.4 kb upstream of the FoxO1A promoter using Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). ChIP analysis of this region of the FoxO1A gene was performed in Cos7 cells, exactly as described previously for analysis of a stably integrated mouse mammary tumor virus promoter (46). Cells were treated with 1 μM dex or vehicle for 30 min before formaldehyde treatment. Primers 5′-atcttcatctaatggtctgg-3′ and 5′-acgaaggtcgggttaag-3′ were amplified −1495 to −1095 upstream of the endogenous FoxO1A promoter.

Preparation of whole-cell extracts and Western analysis

Cell lysates were prepared as described previously (20). In brief, cells were lysed in IPH buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, and fresh 2 mM dithiothreitol, and protease inhibitor cocktail (Roche, Laval, Canada)]. The lysates were sonicated and cleared by centrifugation.
gation. For Western analysis, 20–50 μg whole-cell lysates were resolved by SDS-PAGE. The following antibodies were used for protein detection: αP2 (Cayman Chemical Co., Ann Arbor, MI); actin (H-300), Akt (C-20), PI3KR1 (Z-8), and PI3K p110α (H-239) (Santa Cruz Biotechs, Santa Cruz, CA); IRβ (MA1-22006), FoxO1A (PA1-17036), and FoxO3A (PA1-17028) (Affinity Bioreagents, Golden, CO); IRS1 (06-248) and IRS2 (06-506) (Upstate Biotechnology, Lake Placid, NY); P-Tyr (PY20) (BD Transduction Laboratories, Mississauga, Ontario, Canada); P-IRS1 (Y612) (Biosource, Camarillo, CA); P-Akt (Ser473) (Cell Signaling Technology, Beverly, MA); and Flag (M2).

Signal intensities of Western blots were quantified by measuring densitometry of scanned films using ImageQuant software. Statistical significance was determined using two-tailed, paired Student’s t test in Microsoft Excel.

**Insulin signaling assay**

Cells were stimulated with 1 μM dex or vehicle at confluence for 48 h as described. Cells were then stimulated for 5 min with 100 nM insulin or vehicle in serum-free medium as described under differentiation conditions. Medium was aspirated, and cells were lysed directly in the well by addition of 100 μl cold IPH buffer containing 10% glycerol, 1 mM sodium orthovanadate (Na3VO4), 5 mM sodium pyrophosphate (NaPPi), and 50 mM NaF. Cells were scraped and immediately sonicated for 10 sec at 30% duty cycle. The cellular extracts were cleared by centrifugation at 13,000 × g for 10 min and then immediately denatured by boiling in SDS lysis buffer. Approximately 30 μg of each whole-cell lysate was resolved by SDS-PAGE, and Western analysis was performed.

**Assessment of insulin signaling in adipocytes**

An alternative differentiation protocol was used to assess the effect of glucocorticoids on insulin sensitivity in human adipocytes (29). In brief, sc primary preadipocytes were plated at a density of 3 × 10⁴ cells/cm² in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Burlington, Canada) and antibiotics. The following day (confluence), the cells were induced to differentiate in DMEM supplemented with 10% FBS, antibiotics, 0.85 μM insulin, 100 μM indomethacin, 0.5 μM dex, and 0.25 mM MIX. Thereafter, the medium was not replaced until d 12, at which point differentiated adipocytes were placed in DMEM supplemented with 10% FBS and antibiotics for 2 d and then treated with or without 1 μM dex for 48 h. Two individual donor cells and a pool of five donor preadipocytes were differentiated, respectively. After treatment with dex, the insulin signaling assay was performed exactly as described above. RNA samples were also harvested for RT-PCR analysis.

**Lentiviral infection of human and 3T3-L1 preadipocytes**

All lentiviral system components were purchased from System Biosciences with the exception of the 293FT packaging cell line (Invitrogen). A 2–4 μg pCDH1-MCS1-EF1-paro-FOXO1 expression plasmids using Plus Reagent and Lipofectamine (Invitrogen) per the manufacturer’s instructions. The DNA complex was dropped onto 9 ml 293FT cells in DMEM containing 2% FBS and no antibiotics. The next morning, the medium was changed with 10 ml fresh DMEM containing 2% FBS and no antibiotics. At 48 h after transfection, virus was harvested and passed through a 0.45-μm filter.

**Infection of preadipocytes**

One day before infection, human preadipocytes were seeded either in a 10-cm dish (1 × 10⁶ cells) or in a 12-well dish (90,000 cells). Preadipocytes were infected overnight with a complex of 5 ml virus, 3 ml standard preadipocytes medium, and 4 μg/ml polybrene (10-cm dish) or 600 μl virus, 400 μl medium, and 4 μg/ml polybrene (12-well dish). Medium was replaced the following morning. 3T3-L1 preadipocytes were treated the same as the human preadipocytes, with the following exceptions: 45,000 cells/well of a 12-well dish were seeded and infected with 450 μl virus, 300 μl medium, and 4 μg/ml polybrene overnight.

**Protein visualization by indirect immunofluorescence**

Lentivirally infected human preadipocytes were seeded (50,000 cells per well) into a four-well chamber slide (Nunc, Naperville, IL). Indirect immunofluorescence was performed as previously described (47), using the anti-Flag M2 primary antibody. Images were acquired using a Nikon Eclipse TE300 microscope.

**References**


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