

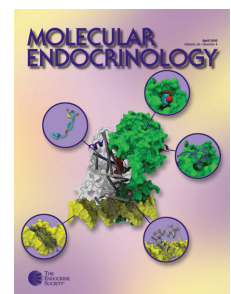
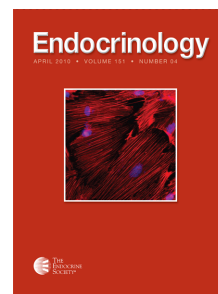
# MOLECULAR ENDOCRINOLOGY

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

Julianna J. Tomlinson, Adèle Boudreau, Dongmei Wu, Houssein Abdou Salem, Amanda Carrigan, Annemarie Gagnon, Alan J. Mears, Alexander Sorisky, Ella Atlas and Robert J. G. Haché

Mol. Endocrinol. 2010 24:104-113 originally published online Nov 3, 2009; , doi: 10.1210/me.2009-0091

To subscribe to *Molecular Endocrinology* or any of the other journals published by The Endocrine Society please go to: <http://mend.endojournals.org/subscriptions/>



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

Julianna J. Tomlinson, Adèle Boudreau, Dongmei Wu, Houssein Abdou Salem, Amanda Carrigan, AnneMarie Gagnon, Alan J. Mears, Alexander Sorisky, Ella Atlas, and Robert J. G. Haché

Departments of Biochemistry, Microbiology, and Immunology (J.J.T., H.A.S., A.C., A.S., R.J.G.H.) and Cellular and Molecular Medicine (A.J.M.) and Medicine (A.J.M., A.S., R.J.G.H.), University of Ottawa, Ottawa, Ontario, Canada K1H 8M5; and Chronic Disease (A.B., D.W., A.G., A.S., E.A., R.J.G.H.) and Vision (A.J.M.) Programs, Ottawa Health Research Institute, Ottawa, Ontario, Canada, K1Y 4E9

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)

**G**lucocorticoids 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 11 $\beta$ -hydroxysteroid dehydrogenase 1 (11 $\beta$ HSD1). There is a

growing recognition from rodent and human correlative studies as well as in murine genetic models that 11 $\beta$ HSD1 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

ISSN Print 0888-8809 ISSN Online 1944-9917  
Printed in U.S.A.

Copyright © 2010 by The Endocrine Society  
doi: 10.1210/me.2009-0091 Received February 23, 2009. Accepted October 7, 2009.  
First Published Online November 3, 2009

Abbreviations: AFC, Average fold change; C/EBP, CCAAT enhancer-binding protein; ChIP, chromatin immunoprecipitation; CI, confidence interval; dex, dexamethasone; FBS, fetal bovine serum; F1DN, dominant-negative construct of FoxO1A; FoxO1A, forkhead box O1A; FDR, false discovery rate; GR, glucocorticoid receptor; GRE, glucocorticoid response element; 11 $\beta$ HSD1, 11 $\beta$ -hydroxysteroid dehydrogenase 1; IR, insulin receptor; IRS, IR substrate; MIX, 3-isobutyl-1-methylxanthine; P-, phospho-; PI3K, phosphoinositide-3-kinase; PI3KR1, PI3K regulatory subunit 1; qRT-PCR, quantitative RT-PCR; RMA, robust multichip average; WAT, white adipose tissue.

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 11 $\beta$ HSD1 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.

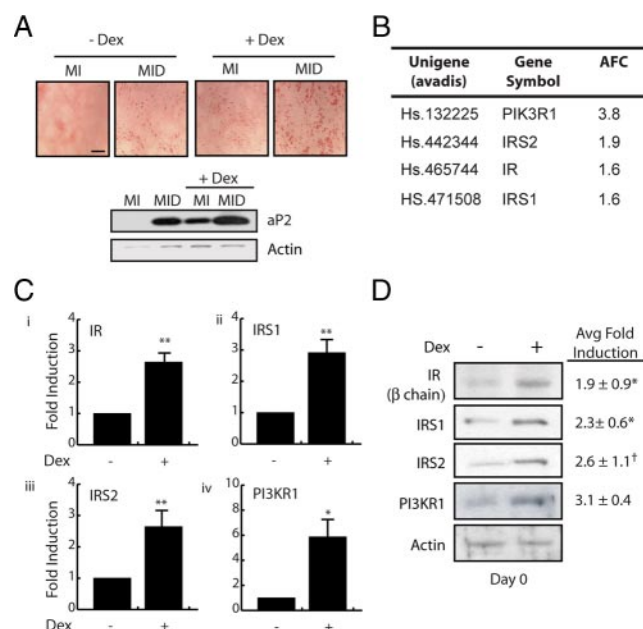
Dex treatment of naive preadipocytes up-regulated key components of the insulin signaling pathway, including IR, IRS1, IRS2, and the p85 $\alpha$  PI3K regulatory subunit, which led the enhancement of protein kinase B (Akt) activation in response to insulin when differentiation was stimulated. These 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 affects 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

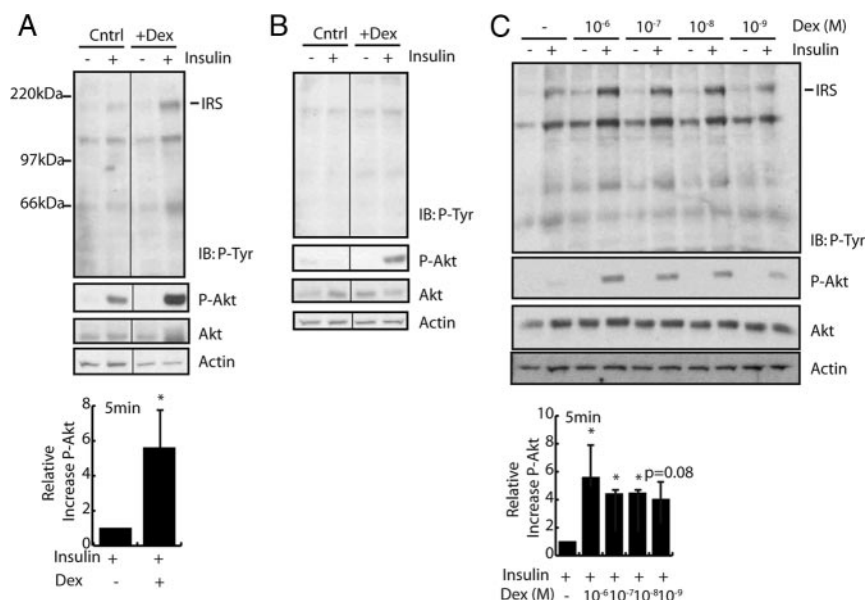
To assess the effect of exposure of preadipocytes to glucocorticoids before the onset of differentiation, we



**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  $\mu$ M dex (*right*, + dex). To initiate differentiation, cells were treated with MIX and insulin (MI) or MIX, insulin, and 1  $\mu$ M 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 d 0 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  $\pm$  SEM. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$  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  $\pm$  SD was calculated by densitometry using ImageQuant software ( $n \geq 3$ ).  $P$  value was determined as described in **C**:  $\dagger$ ,  $P \leq 0.1$ ; \*,  $P \leq 0.05$ . Hs., *Homo sapiens*.

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 and expression of the late adipogenic marker aP2 (Fig. 1A). Moreover, dex pretreatment provided for an enhancement of subsequent differentiation that was in addition to the effect of the dex included in the differentiation cocktail, suggesting separate or complementary signaling targets.

To investigate the transcriptional consequences of the dex pretreatment of the preadipocytes, we performed an Affymetrix microarray analysis of RNA prepared from pools of primary human preadipocytes. For this analysis, changes in gene expression patterns were quantified using both robust multichip average (RMA) and GC-RMA



**FIG. 2.** Pretreatment of human preadipocytes with physiological concentrations of glucocorticoids results in increased insulin sensitivity. A and B, Vehicle- (Cntrl) and dex-pretreated human preadipocytes (d 0) were stimulated with 100 nM insulin in serum-free, chemically defined medium for 5 min (A) or 1 h (B) as indicated. Western analysis of P-Tyr residues using a pan-P-Tyr antibody (P-Tyr) (top immunoblot) revealed major bands that overlaid with the IRS proteins (IRS). Immunoblots (IB) were serially reprobed with antibodies against active phosphorylated at S473 Akt (P-Akt), total cellular Akt, and actin. Quantification of the P-Akt signal in A was normalized to total cellular Akt and plotted as relative fold induction  $\pm$ SD by dex treatment where  $n = 5$ . C, Confluent preadipocytes were pretreated with vehicle or  $10^{-6}$  to  $10^{-9}$  M dex as indicated for 48 h, with the level of P-Tyr and P-Akt determined 5 min subsequent to stimulation with 100 nM insulin. Fold induction of P-Akt levels was quantified as in A where  $n = 3$ . \*,  $P \leq 0.05$ .

analysis followed by false discovery rate (FDR)-confidence interval (CI)-based statistical validation (24, 25). In these experiments, C/EBP $\delta$ , 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.

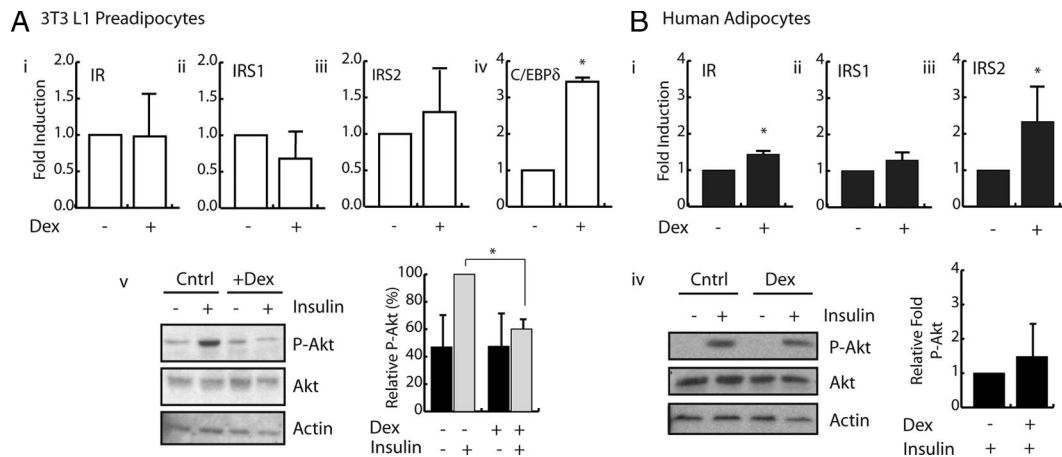
Interestingly and somewhat unexpectedly, analysis of the microarray data showed 1.6- to 3.8-fold enrichment in the mRNAs for key components of the insulin signaling pathway, IR, IRS1, IRS2, and PI3K regulatory subunit 1 (PI3KR1), when preadipocytes were treated with dex (Fig. 1B). Quantitative RT-PCR (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 also 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 ( $\sim$ 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, P-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,  $P \leq 0.05$ ) 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/EBP $\delta$ , a known direct transcriptional target of glucocorticoids in 3T3-L1 preadipocytes was induced  $3.4 \pm 0.2$ -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



**FIG. 3.** The effects of glucocorticoids on insulin sensitivity are specific to human preadipocytes. Fold induction of *Ir* (i), *Irs1* (ii), *Irs2* (iii), and *Cebpd* (iv) mRNA abundance in 3T3-L1 preadipocytes (A) and human adipocytes (B) after 48 h treatment with vehicle (–) or 1  $\mu$ M dex (+) as described in the *Materials and Methods*. Data represent average fold induction  $\pm$ SEM where  $n \geq 3$  performed in duplicate as described in Fig. 2. A, panel v, and B, panel iv, Western analysis of Akt-S473 phosphorylation (P-Akt) in response to 5 min stimulation with 100 nM insulin after 48 h pretreatment with vehicle (Cntrl) or 1  $\mu$ M dex (+Dex). Quantification and statistical analysis was performed as described in Fig. 2A, where  $n = 3$  (A) and  $n = 6$  (B). \*,  $P \leq 0.05$ .

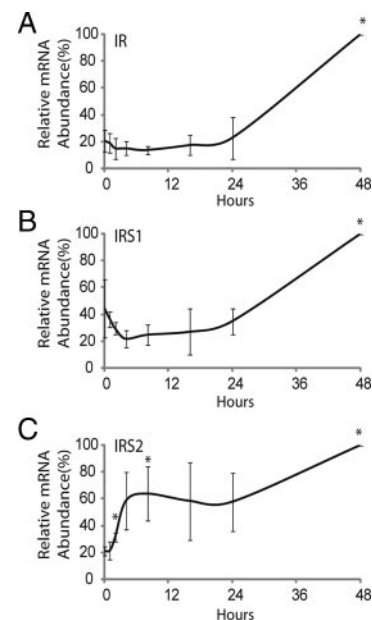
glucocorticoid treatment in primary human adipocytes that had been differentiated in culture under optimal adipogenic conditions for 12 d (29) and withdrawn from glucocorticoids for 48 h before restimulation (Fig. 3B). IRS1 induction was completely abrogated, whereas IR induction was markedly reduced 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

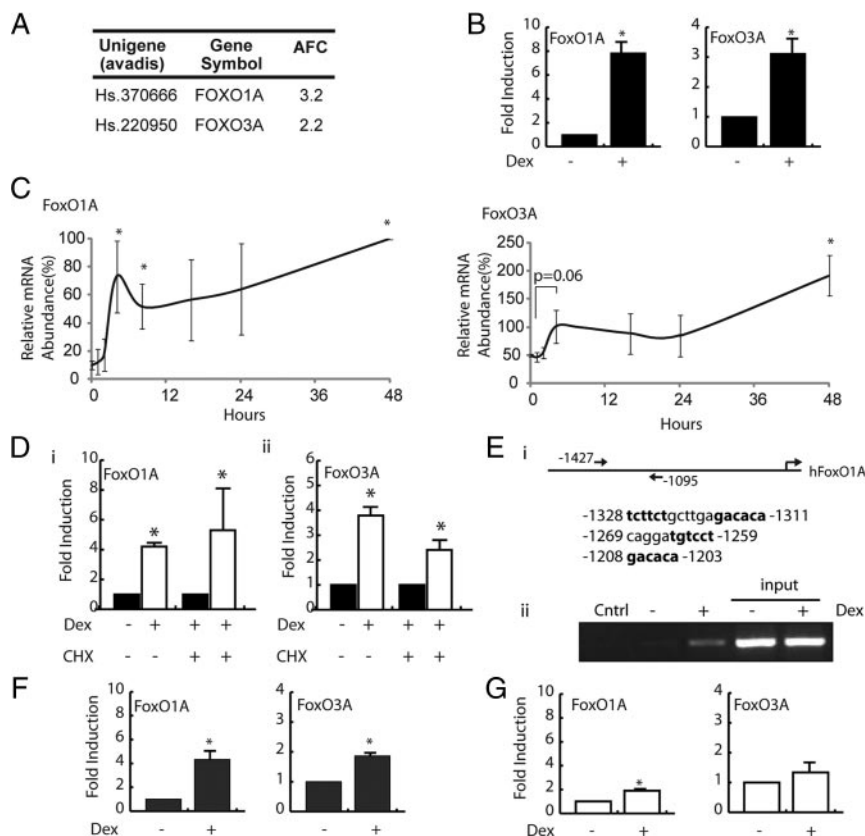
Time-course analysis of IR, IRS1, and IRS2 induction in human preadipocytes revealed a rapid 3-fold induction of IRS2 mRNA within 4 h of dex treatment of primary human preadipocytes, whereas the induction of IR and IRS1 were delayed for at least 24 h (Fig. 4). The rapid IRS2 response was consistent with the previous report of glucocorticoid response elements (GREs) that mediated glucocorticoid- and progestin-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>).



**FIG. 4.** IR and IRS1 are not primary transcriptional targets of glucocorticoid action in human preadipocytes. *IR* (A), *IRS1* (B), and *IRS2* (C) mRNA induction profile after continuous stimulation with 1  $\mu$ M dex. Human primary preadipocytes were stimulated with dex upon reaching confluence as previously described. The time point of 48 h after stimulation represents when the cells would have otherwise been induced to differentiate (day 0). mRNA abundance was quantified by qRT-PCR where  $n = 3$ . \*,  $P \leq 0.05$ .



**FIG. 5.** FoxO transcription factors are induced in response to glucocorticoids. **A**, Microarray analysis of glucocorticoid-dependent induction of *FOXO1A* and *FOXO3A* mRNA expression after 48 h pretreatment of human primary preadipocytes. AFC of mRNA expression compared with control cells was determined using RMA analysis, and statistical validation was calculated using FDR-CI analysis. **B**, qRT-PCR analysis of AFC of *FOXO1A* and *FOXO3A* mRNA expression after 48 h treatment with dex in confluent human preadipocytes. **C**, *FOXO1A* and *FOXO3A* mRNA induction profile in human preadipocytes after continuous stimulation with 1  $\mu$ M dex as described in Fig. 4. **D**, qRT-PCR analysis of *FOXO1A* and *FOXO3A* mRNA abundance in confluent human primary preadipocytes after 8 h treatment with vehicle, 1  $\mu$ M dex, and 2  $\mu$ g/ml cycloheximide (CHX) as indicated. **E**, **i**, Location of ChIP primer annealing sites and predicted GREs upstream of the FoxO1A promoter; **ii**, ChIP of GR occupancy after 30 min treatment with vehicle (-) or 1  $\mu$ M dex (+) where control (Ctrl) represents PCR conditions with no template DNA. Results are representative of three repeats. **F** and **G**, qRT-PCR analysis of AFC of *FOXO1A* and *FOXO3A* mRNA in human adipocytes (**F**) and 3T3-L1 preadipocytes (**G**). Data are represented  $\pm$  SEM, where  $n = 3$  as described in Fig. 2. \*,  $P \leq 0.05$ .

The qRT-PCR analysis set *FOXO1A* mRNA induction after glucocorticoid treatment at  $7.9 \pm 1.0$ -fold and *FOXO3A* mRNA at  $3.1 \pm 0.7$ -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

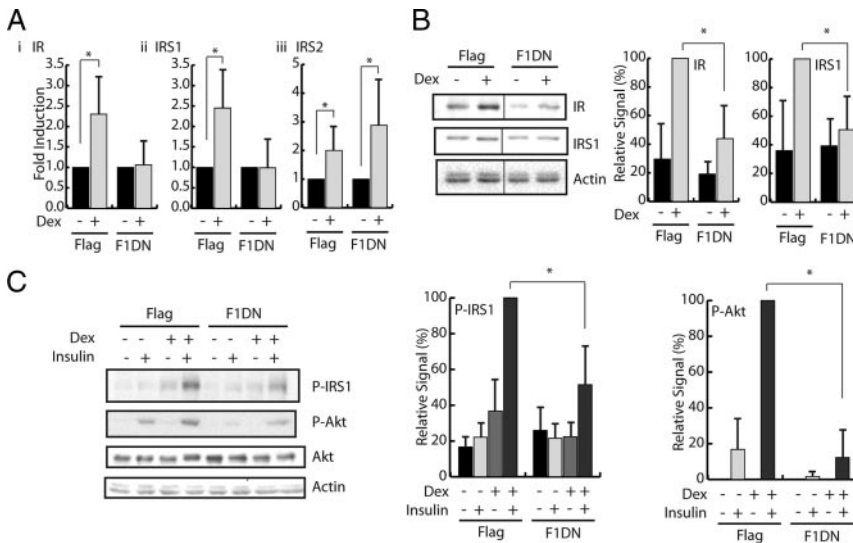
the region between 1.1 and 1.4 kb upstream of the promoter within 30 min of dex treatment (Fig. 5E).

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 ( $1.9 \pm 0.2$ -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



**FIG. 6.** FoxO1A mediates glucocorticoid-dependent insulin sensitization of human primary preadipocytes. **A**, Relative fold induction by dex of *IR* (i), *IRS1* (ii), and *IRS2* (iii) mRNA abundance after 48 h pretreatment with 1  $\mu$ M dex in human preadipocytes infected with empty virus (Flag) or with F1DN-expressing virus. Data are plotted  $\pm$ SEM, where  $n = 5$ . **B**, Western analysis of IR and IRS1 protein expression after stimulation with dex as described in **A**. Signal intensity was measured as described in Fig. 1, where  $n = 4$ . **C**, Western analysis of insulin signaling pathway activation in human preadipocytes in control (Flag) and F1DN-expressing cells, where  $n = 4$ . Assay and quantification was performed as described in Fig. 2. \*,  $P \leq 0.05$ . P-Akt, Phosphorylated at S473 Akt.

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 11 $\beta$ HSD1 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/EBP $\delta$ , C/EBP $\beta$ , 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/EBP $\delta$ , a direct target of GR that contributes to subsequent differentiation. However, the glucocorticoids coin-

cently induced the dominant-negative C/EBP, CHOP, to a similar level and the subsequent profile of C/EBP $\delta$  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/EBP $\beta$  and C/EBP $\delta$  until it is down-regulated 12–24 h after the stimulation of differentiation (38). Furthermore, C/EBP $\delta$  also was up-regulated by dex in 3T3-L1 preadipocytes without affecting insulin sensitivity. Thus, the up-regulation of C/EBP $\delta$  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.

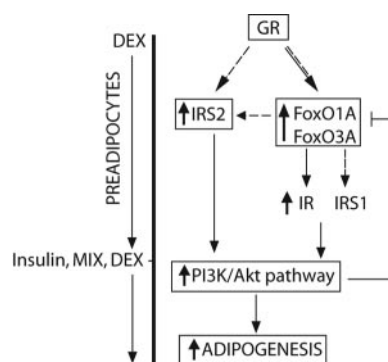
FoxO transcription factors are regulated both transcriptionally and through posttranslational modifications that control their subcellular localization (43). Thus, the delay between induction of FoxO1A/3A and IR/IRS1 in response to dex may reflect subsequent events required for FoxO1A/3A to require transcriptional 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 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 GR<sup>dim/dim</sup> 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 sub-



**FIG. 7.** Proposed model of molecular mechanisms through which pretreatment with glucocorticoids leads to increased insulin sensitivity and subsequent differentiation of human primary preadipocytes. Glucocorticoids lead to the up-regulation of IRS2, FoxO1A, and FoxO3A and subsequent FoxO-dependent activation of IR and IRS1. *Dashed lines* indicate an upstream transcriptional regulatory relationship for which further studies are required to confirm whether these relationships are direct.

sequent 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/EBP $\delta$  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 11 $\beta$ HSD1 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 *Swa*I and *Bam*HI restriction sites.

### Cell maintenance and differentiation

Cryopreserved, sc human primary preadipocytes from female donors with a normal body mass index ( $22.5 \pm 0.2 \text{ kg/m}^2$ ) were purchased from Zen-Bio Inc. (Research Triangle Park, NC). Cells were maintained at 5% CO<sub>2</sub> 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% CO<sub>2</sub> 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  $\mu\text{M}$  dex or vehicle in growth medium containing 3% calf serum for 48 h. Thereafter (d 0), cells were differentiated under serum-free conditions or the preadipocytes were harvested for protein or mRNA analysis or were used for the insulin signaling assay.

For differentiation, on d 0, the medium was replaced with serum-free DMEM-Ham's F12 (1:1, vol/vol) medium supplemented with 33  $\mu\text{M}$  biotin, 17  $\mu\text{M}$  pantothenate, 10  $\mu\text{g/ml}$  transferrin, 0.2 nM T<sub>3</sub>, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 U/ml nystatin. To stimulate differentiation, the cells were treated with 100 nM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (MIX) from d 0–4, 1  $\mu\text{M}$  dex for the initial 48 h, and 5  $\mu\text{M}$  troglitazone from d 2–4. On d 4 and every 3–4 d thereafter, the medium was replaced with serum-free medium containing 100 nM insulin until d 8, after which the medium was not replaced.

Each experiment using human preadipocytes was conducted using at least one pool of preadipocytes from five individual donors and repeated using pooled cultures from two, three, or five donors and/or preadipocytes from one donor.

### Oil Red O staining of neutral lipid content in mature adipocytes

To assess neutral lipid content, mature adipocytes were washed twice with PBS and fixed with 2 ml 10% formalin in PBS for 1 h. Cells were then washed once with PBS, stained with 2 ml Oil Red O for 2 h, and then washed with distilled water (44). Photomicrographs of Oil Red O-stained cells were taken with a Leica MZ125 microscope and represent approximately 85% of a well in a 12-well dish.

### Microarray analysis of gene expression profiles in human primary preadipocytes

#### Sample preparation

Human sc primary preadipocytes from five female donors (average body mass index  $22.5 \pm 0.2 \text{ kg/m}^2$ ) were pooled before

the initial seeding. Cells were maintained and treated with dex upon reaching confluence exactly as described above. After 48 h stimulation with 1  $\mu\text{M}$  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  $-80 \text{ C}$  for future RT-PCR-based validation of microarray results.

#### Microarray analysis

Microarray processing was performed by StemCore (Ottawa, Canada) using the Affymetrix (Santa Clara, CA) human genome U133 Plus 2.0 GeneChip array. One of the control and treated arrays was disregarded in the analysis due to failing internal quality control standards. As a result, analysis was performed on duplicate samples. The raw data were first analyzed using Affymetrix Microarray Suite version 5.0 (MAS5) to establish present and absent calls for each probe set. Probe sets that were absent on all four arrays were not included in further analysis. The data were then normalized, and the signal intensities were calculated using both RMA and GC-RMA. Statistical relevance was determined for each probe set based on an AFC of at least 1.5 using the FDR-CI method as previously described (24, 25). Only probe sets that had a statistically relevant AFC of at least 1.5 by both RMA and GC-RMA algorithms were considered.

#### 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  $\beta$ -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  $\pm$ 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  $\mu\text{M}$  dex, and 2  $\mu\text{g/ml}$  cycloheximide as indicated for 8 h. Cells treated with cycloheximide were pulsed with 2  $\mu\text{g/ml}$  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  $\mu\text{M}$  dex or vehicle for 30 min before formaldehyde treatment. Primers 5'-atctccatcctaagtgtccc-3' and 5'-acggaagtgatcgggtgtaag-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  $\mu\text{g}$  whole-cell lysates were resolved by SDS-PAGE. The following antibodies were used for protein detection: aP2 (Cayman Chemical Co., Ann Arbor, MI); actin (H-300), Akt (C-20), PI3KR1 (Z-8), and PI3K p110 $\alpha$  (H-239) (Santa Cruz Biotechnologies, Santa Cruz, CA); IR $\beta$  (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  $\mu\text{M}$  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  $\mu\text{l}$  cold IPH buffer containing 10% glycerol, 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ), 5 mM sodium pyrophosphate ( $\text{NaPPi}$ ), 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  $13,000 \times g$  for 10 min and then immediately denatured by boiling in SDS lysis buffer. Approximately 30  $\mu\text{g}$  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  $3 \times 10^4$  cells/cm<sup>2</sup> 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  $\mu\text{M}$  insulin, 100  $\mu\text{M}$  indomethacin, 0.5  $\mu\text{M}$  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  $\mu\text{M}$  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).

### Generation of virus

293FT cells maintained in DMEM high glucose supplemented with 10% FBS, 2 mM L-glutamine, MEM nonessential amino acids, and 500 mg/ml G418, and  $3.75 \times 10^6$  cells were seeded into a 10-cm dish in regular medium lacking G418. Cells were transfected with 10  $\mu\text{g}$  pPACKH1 packaging plasmids and

2–4  $\mu\text{g}$  pCDH1-MCS1-EF1-puro-FOXO1 expression plasmids using Plus Reagent and Lipofectamine (Invitrogen) as 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- $\mu\text{m}$  filter.

### Infection of preadipocytes

One day before infection, human preadipocytes were seeded either in a 10-cm dish ( $1 \times 10^6$  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  $\mu\text{g}/\text{ml}$  polybrene (10-cm dish) or 600  $\mu\text{l}$  virus, 400  $\mu\text{l}$  medium, and 4  $\mu\text{g}/\text{ml}$  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  $\mu\text{l}$  virus, 300  $\mu\text{l}$  medium, and 4  $\mu\text{g}/\text{ml}$  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.

### Acknowledgments

Address all correspondence and requests for reprints to: Dr. Robert Haché, Department of Biological Sciences, 2500 University Drive Northwest, University of Calgary, Calgary, Alberta, Canada T2N 1N4. E-mail: rjhache@ucalgary.ca.

This work was supported by the Heart and Stroke Foundation of Ontario (Grant T5813) to R.J.G.H and supported in part by a Canadian Institutes of Health Research grant to A.S. J.J.T. was supported by combined Heart and Stroke, Canadian Diabetes Association, and Canadian Institutes of Health Research Studentship.

R.J.G.H. was a University of Ottawa Health Research Chair.

Present address for J.J.T.: Neuroscience Program, Ottawa Health Research Institute, Ottawa, Ontario, Canada K1Y 4E9.

Disclosure Summary: The authors have nothing to disclose.

### References

1. Andrews RC, Walker BR 1999 Glucocorticoids and insulin resistance: old hormones, new targets. *Clin Sci (Lond)* 96:513–523
2. Björntorp P, Rosmond R 2000 Obesity and cortisol. *Nutrition* 16: 924–936
3. Boscaro M, Barzon L, Fallo F, Sonino N 2001 Cushing's syndrome. *Lancet* 357:783–791
4. McDonough AK, Curtis JR, Saag KG 2008 The epidemiology of glucocorticoid-associated adverse events. *Curr Opin Rheumatol* 20:131–137
5. Walker BR, Andrew R 2006 Tissue production of cortisol by 11 $\beta$ -hydroxysteroid dehydrogenase type 1 and metabolic disease. *Ann NY Acad Sci* 1083:165–184
6. Wake DJ, Walker BR 2004 11 $\beta$ -Hydroxysteroid dehydrogenase type 1 in obesity and the metabolic syndrome. *Mol Cell Endocrinol* 215:45–54

7. Burén J, Liu HX, Jensen J, Eriksson JW 2002 Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. *Eur J Endocrinol* 146:419–429
8. Lundgren M, Burén J, Ruge T, Myrnäs T, Eriksson JW 2004 Glucocorticoids down-regulate glucose uptake capacity and insulin-signaling proteins in omental but not subcutaneous human adipocytes. *J Clin Endocrinol Metab* 89:2989–2997
9. Turnbow MA, Keller SR, Rice KM, Garner CW 1994 Dexamethasone down-regulation of insulin receptor substrate-1 in 3T3-L1 adipocytes. *J Biol Chem* 269:2516–2520
10. Gregoire FM, Smas CM, Sul HS 1998 Understanding adipocyte differentiation. *Physiol Rev* 78:783–809
11. Taniguchi CM, Emanuelli B, Kahn CR 2006 Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85–96
12. Rosen ED, MacDougald OA 2006 Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* 7:885–896
13. Blüher M, Michael MD, Peroni OD, Ueki K, Carter N, Kahn BB, Kahn CR 2002 Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev Cell* 3:25–38
14. Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, Coleman KG 2003 Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKCB. *J Clin Invest* 112:197–208
15. Laustsen PG, Michael MD, Crute BE, Cohen SE, Ueki K, Kulkarni RN, Keller SR, Lienhard GE, Kahn CR 2002 Lipotrophic diabetes in *Irs1<sup>-/-</sup>/Irs3<sup>-/-</sup>* double knockout mice. *Genes Dev* 16:3213–3222
16. Rui L, Fisher TL, Thomas J, White MF 2001 Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2. *J Biol Chem* 276:40362–40367
17. Magun R, Burgering BM, Coffey PJ, Pardasani D, Lin Y, Chabot J, Sorisky A 1996 Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. *Endocrinology* 137:3590–3593
18. Sakaue H, Ogawa W, Matsumoto M, Kuroda S, Takata M, Sugimoto T, Spiegelman BM, Kasuga M 1998 Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase. *J Biol Chem* 273:28945–28952
19. Yeh WC, Cao Z, Classon M, McKnight SL 1995 Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev* 9:168–181
20. Tomlinson JJ, Boudreau A, Wu D, Atlas E, Haché RJ 2006 Modulation of early human preadipocyte differentiation by glucocorticoids. *Endocrinology* 147:5284–5293
21. Wiper-Bergeron N, Salem HA, Tomlinson JJ, Wu D, Haché RJ 2007 Glucocorticoid-stimulated preadipocyte differentiation is mediated through acetylation of C/EBP $\beta$  by GCN5. *Proc Natl Acad Sci USA* 104:2703–2708
22. Wiper-Bergeron N, Wu D, Pope L, Schild-Poulter C, Haché RJ 2003 Stimulation of preadipocyte differentiation by steroid through targeting of an HDAC1 complex. *EMBO J* 22:2135–2145
23. Farmer SR 2006 Transcriptional control of adipocyte formation. *Cell Metab* 4:263–273
24. Hero AO, Fleury G, Mears A, Swaroop A 2004 Multicriteria gene screening for analysis of differential expression with DNA microarrays. *J Applied Signal Process* 2004:43–52
25. Yoshida S, Mears AJ, Friedman JS, Carter T, He S, Oh E, Jing Y, Farjo R, Fleury G, Barlow C, Hero AO, Swaroop A 2004 Expression profiling of the developing and mature *Nrl<sup>-/-</sup>* mouse retina: identification of retinal disease candidates and transcriptional regulatory targets of *Nrl*. *Hum Mol Genet* 13:1487–1503
26. Kim YB, Peroni OD, Franke TF, Kahn BB 2000 Divergent regulation of Akt1 and Akt2 isoforms in insulin target tissues of obese Zucker rats. *Diabetes* 49:847–856
27. Sasaoka T, Ishiki M, Sawa T, Ishihara H, Takata Y, Imamura T, Usui I, Olefsky JM, Kobayashi M 1996 Comparison of the insulin and insulin-like growth factor 1 mitogenic intracellular signaling pathways. *Endocrinology* 137:4427–4434
28. Redei EE 2008 Molecular genetics of the stress-responsive adrenocortical axis. *Ann Med* 40:139–148
29. Artemenko Y, Gagnon A, Aubin D, Sorisky A 2005 Anti-adipogenic effect of PDGF is reversed by PKC inhibition. *J Cell Physiol* 204:646–653
30. Ide T, Shimano H, Yahagi N, Matsuzaka T, Nakakuki M, Yamamoto T, Nakagawa Y, Takahashi A, Suzuki H, Sone H, Toyoshima H, Fukamizu A, Yamada N 2004 SREBPs suppress IRS-2-mediated insulin signalling in the liver. *Nat Cell Biol* 6:351–357
31. Vassen L, Wegrzyn W, Klein-Hitpass L 1999 Human insulin receptor substrate-2 (IRS-2) is a primary progesterone response gene. *Mol Endocrinol* 13:485–494
32. Puig O, Marr MT, Ruhf ML, Tjian R 2003 Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* 17:2006–2020
33. Puig O, Tjian R 2005 Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. *Genes Dev* 19:2435–2446
34. Waddell DS, Baehr LM, van den Brandt J, Johnsen SA, Reichardt HM, Furlow JD, Bodine SC 2008 The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *Am J Physiol Endocrinol Metab* 295:E785–E797
35. Nakae J, Kitamura T, Silver DL, Accili D 2001 The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* 108:1359–1367
36. Pantoja C, Huff JT, Yamamoto KR 2008 Glucocorticoid signaling defines a novel commitment state during adipogenesis in vitro. *Mol Biol Cell* 19:4032–4041
37. Smas CM, Chen L, Zhao L, Latasa MJ, Sul HS 1999 Transcriptional repression of pref-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation. *J Biol Chem* 274:12632–12641
38. Tang QQ, Lane MD 2000 Role of C/EBP homologous protein (CHOP-10) in the programmed activation of CCAAT/enhancer-binding protein- $\beta$  during adipogenesis. *Proc Natl Acad Sci USA* 97:12446–12450
39. Nakae J, Oki M, Cao Y 2008 The FoxO transcription factors and metabolic regulation. *FEBS Lett* 582:54–67
40. Jing E, Gesta S, Kahn CR 2007 SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. *Cell Metab* 6:105–114
41. Nakae J, Cao Y, Oki M, Orba Y, Sawa H, Kiyonari H, Iskandar K, Suga K, Lombes M, Hayashi Y 2008 Forkhead transcription factor FoxO1 in adipose tissue regulates energy storage and expenditure. *Diabetes* 57:563–576
42. Nakae J, Kitamura T, Kitamura Y, Biggs 3rd WH, Arden KC, Accili D 2003 The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Developmental cell* 4:119–129
43. Van Der Heide LP, Hoekman MF, Smidt MP 2004 The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem J* 380:297–309
44. Schwarz EJ, Reginato MJ, Shao D, Krakow SL, Lazar MA 1997 Retinoic acid blocks adipogenesis by inhibiting C/EBP $\beta$ -mediated transcription. *Mol Cell Biol* 17:1552–1561
45. Gorzelnik K, Janke J, Engeli S, Sharma AM 2001 Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. *Horm Metab Res* 33:625–627
46. Carrigan A, Walther RF, Salem HA, Wu D, Atlas E, Lefebvre YA, Haché RJ 2007 An active nuclear retention signal in the glucocorticoid receptor functions as a strong inducer of transcriptional activation. *J Biol Chem* 282:10963–10971
47. Walther RF, Atlas E, Carrigan A, Rouleau Y, Edgecombe A, Visentin L, Lamprecht C, Addicks GC, Haché RJ, Lefebvre YA 2005 A serine/threonine-rich motif is one of three nuclear localization signals that determine unidirectional transport of the mineralocorticoid receptor to the nucleus. *J Biol Chem* 280:17549–17561