Differential Modulation of 3T3-L1 Adipogenesis Mediated by 11β-Hydroxysteroid Dehydrogenase-1 Levels*3

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The localized activation of circulating glucocorticoids in vivo by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays a critical role in the development of the metabolic syndrome. However, the precise contribution of 11β-HSD1 in the initiation of adipogenesis by inactive glucocorticoids is not fully understood. 3T3-L1 fibroblasts can be terminally differentiated to mature adipocytes in a glucocorticoid-dependent manner. Both inactive rodent dehydrocorticosterone and human cortisone were able to substitute for the synthetic glucocorticoid dexamethasone in 3T3-L1 adipogenesis, suggesting a potential role for 11β-HSD1 in these effects. Differentiation of 3T3-L1 cells caused a strong increase in 11β-HSD1 protein levels, which occurred late in the differentiation protocol. Reduction of 11β-HSD1 activity in 3T3-L1 fibroblasts, achieved by pharmacological inhibition or adenovirally mediated delivery of short hairpin RNA constructs, specifically blocked the ability of inactive glucocorticoids to drive 3T3-L1 differentiation. However, even modest increases in exogenous 11β-HSD1 expression in 3T3-L1 fibroblasts, to levels comparable with endogenous 11β-HSD1 in differentiated 3T3-L1 adipocytes, were sufficient to block adipogenesis. Luciferase reporter assays indicated that overexpressed 11β-HSD1 was catalyzing the inactivating dehydrogenase reaction, because the ability of both active and inactive glucocorticoids to activate the glucocorticoid receptor were largely suppressed. These results suggest that the temporal regulation of 11β-HSD1 expression is tightly controlled in 3T3-L1 cells, so as to mediate the initiation of differentiation by inactive glucocorticoids and also to prevent the inhibitory activity of prematurely expressed 11β-HSD1 during adipogenesis.

Glucocorticoids are steroid hormones that have vital physiological effects in many tissues throughout the body, including adipose tissue. They are secreted by the adrenal cortex under the hormonal regulation of the hypothalamic–pituitary–adrenal axis and circulate in both active and inactive forms in rodents and humans. In stressful situations, glucocorticoid secretion increases up to 10-fold, resulting in enhanced cardiac function, increased intravascular pressure, improved skeletal muscle work capacity, and more efficient energy mobilization (1, 2), enabling the human body to survive during acutely adverse situations. However, chronic glucocorticoid excess can be deleterious, as exemplified in Cushing’s syndrome, which is characterized by endogenous glucocorticoid overproduction. These individuals develop a reversible visceral obesity (3), as well as insulin resistance, hypertension, and dyslipidemia (4). Similarly, patients treated for prolonged periods with exogenous glucocorticoids can develop central adiposity and its complications (5), whereas reduction of glucocorticoid production or administration reverses these effects (5). Similar metabolic abnormalities are found in humans with the clinical entity that has been dubbed the metabolic syndrome (6); thus, it was hypothesized that there may be a potential link between glucocorticoid action and the development of the metabolic syndrome in humans.

Unexpectedly, humans with idiopathic obesity and/or the metabolic syndrome were found to have normal or even low circulating glucocorticoid levels (7). However, glucocorticoids circulate in both active (cortisone in mice and cortisol in humans) and inactive (dehydrocorticosterone in mice and cortisone in humans) forms. Two enzymes have been described that mediate the localized interconversion of circulating glucocorticoids in vivo. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1),3 was cloned from rat liver and was subsequently detected in a wide range of other tissues, especially adipose and central nervous system (8). The type 2 isomorph (11β-HSD2) was found to have only 20% amino acid homology to 11β-HSD1 (9, 10) and was reported to be expressed in mineralocorticoid target tissues, such as the kidney, as well as colon, placenta, and vascular endothelium (9, 11–14). 11β-HSD1 is bidirectional but acts predominantly as an oxoreductase in vivo to generate active from inactive glucocorticoid (15, 16). The type 2 isomorph has been found to have only dehydrogenase activity, converting active glucocorticoids to their inactive forms (17–19).

Recent advances in our understanding of the localization of glucocorticoids in vivo have provided evidence supporting a potential link between glucocorticoid action and the development of the metabolic syndrome in humans. This paper is dedicated to the memory of Dr. Jaime Kim (1972–2005).
glucocorticoid interconversion on global glucose and lipid metabolism. 11β-HSD1 knock-out mice have low intracellular glucocorticoid levels and are protected from stress- or obesity-induced hyperglycemia (20). These mice also have lower triglyceride levels, increased high density lipoprotein cholesterol levels, and improved glucose tolerance and hepatic insulin sensitivity (21). Conversely, when 11β-HSD1 was selectively overexpressed in adipose tissue in transgenic mice, there was a 2–3-fold increase in the local tissue concentration of active corticosterone (22). The animals also had disproportionately high visceral fat relative to total body fat, which correlated with the induction of insulin resistance, glucose intolerance, and dyslipidemia. Thus, selective modulation of 11β-HSD1 activity impacted localized glucocorticoid conversion and insulin sensitivity in a variety of animal models.

Although the role of 11β-HSD1 in mature adipocytes has been well established, less is known about the activity of the enzyme in the preadipocyte, primarily because of the use of the aP2 promoter in transgenic lines, which drives expression of the exogenous gene near the terminal phase of adipocytic differentiation. The critical role of glucocorticoids in the initiation of adipogenesis has been demonstrated in vitro by utilizing preadipocyte 3T3-L1 and 3T3-F442A cell lines as well as primary cultures of stromal precursor cells from adipose tissue (23, 24). Additionally, the 11β-HSD1 pharmacological inhibitor 18β-glycyrrhetinic acid (GE) blocked stromal cell differentiation by the inactive human glucocorticoid cortisone in vitro, suggesting a potential role for the enzyme in the localized activation of glucocorticoids in preadipocyte cells (24). In the present study, we directly modulated 11β-HSD1 enzymes levels in 3T3-L1 fibroblasts through the use of adenoovirally mediated protein overexpression and delivery of shRNA constructs. We found that 11β-HSD1 is critical for the induction of adipocyte differentiation by inactive glucocorticoids. However, enzymatic activity must be tightly maintained in a narrow range in fibroblasts, because overexpression of 11β-HSD1 to levels comparable with those present in 3T3-L1 adipocytes blocked glucocorticoid receptor activation and 3T3-L1 differentiation driven by both inactive and active glucocorticoids.

**EXPERIMENTAL PROCEDURES**

3T3-L1 Cell Culture and Differentiation—3T3-L1 fibroblasts were cultured as previously described (25). Two days after reaching confluency, differentiation was initiated by the addition of DMEM (Mediatech, Herndon, VA) containing 10% fetal bovine serum (FBS; Hyclone, Logan UT; Biocrest, Cedar Crest, TX), 167 mM porcine insulin, and 0.5 mM isobutylmethyl xanthine (MIx; both from Sigma) in the absence or presence of the thione (MIX; both from Sigma). After 3 days, the medium was removed, and the cells were cultured for two more days in DMEM plus 10% FBS and 167 mM insulin. The cells were then maintained in DMEM plus 10% FBS medium until use, usually 1–3 days after completion of the differentiation protocol. In some experiments, 5 μM GE (Sigma) was also included in the initial 3-day incubation with various glucocorticoids. Oil Red O (Sigma) staining of 3T3-L1 adipocytes was performed as described (26).

**11β-HSD1 Levels Regulate 3T3-L1 Differentiation**

**Sequencing of Adipocytic and Hepatic Mouse 11β-HSD1—**

Primers were designed from the highly referenced, published sequence for hepatic mouse 11β-HSD1 (GenBank™ accession number NM_008288). To generate a FLAG-tagged adenoviral construct, the following primers (ITD, Coralville, IA) were used: 5’ sense, GGA TCC ACC ATG GAC TAT AAA GAC GAC GAC GAC AAA ATG GCA GTT ATG AAA; 3’ antisense, GGC GCC GC CTA GTT ACT AAC CAT. Replicate PCRs (final volume, 50 μl) were run using 2.5 ng of murine hepatic or adipocytic cDNA library (Biochain Institute Inc., Hayward, CA), 100 pmol of each primer, and 400 μM dNTPs in 60 mM Tris, pH 9.0, 15 mM ammonium sulfate, 2 mM MgCl₂. The following cycling conditions were used to amplify 11β-HSD1: 2 min at 95°C; 30 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1.5 min; and a 7-min extension at 72°C. PCRs were analyzed on 1% agarose gels, and the single ~900-bp product for each reaction was subcloned into the TOPO TA vector according to the supplier’s instructions (Invitrogen). Colonies positive for insert were purified and fully sequenced in both directions using the University of Chicago Cancer Core Sequencing Facility. During multiple PCRs using different lots of adipocytic cDNA library, four nonconservative changes from the hepatic 11β-HSD1 sequence were consistently obtained (NM_008288 sequence - adipocyte sequence, corresponding amino acid change): bp 41–43 TCC (GenBank™ accession number NM_008288) to levels 3GAC GAC GAC AAA ATG GCA GTT ATG AAA; 3’ antisense, GGC GCC GC CTA GTT ACT AAC CAT. Replicate PCRs (final volume, 50 μl) were run using 2.5 ng of murine hepatic cDNA library (Biochain Institute Inc., Hayward, CA), 100 pmol of each primer, and 400 μM dNTPs in 60 mM Tris, pH 9.0, 15 mM ammonium sulfate, 2 mM MgCl₂. The following cycling conditions were used to amplify 11β-HSD1: 2 min at 95°C; 30 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1.5 min; and a 7-min extension at 72°C. PCRs were analyzed on 1% agarose gels, and the single ~900-bp product for each reaction was subcloned into the TOPO TA vector according to the supplier’s instructions (Invitrogen). Colonies positive for insert were purified and fully sequenced in both directions using the University of Chicago Cancer Core Sequencing Facility. During multiple PCRs using different lots of adipocytic cDNA library, four nonconservative changes from the hepatic 11β-HSD1 sequence were consistently obtained (NM_008288 sequence - adipocyte sequence, corresponding amino acid change): bp 41–43 TCC → TTC, Ser → Phe; bp 694–696 GAC → AAC, Asp → Asn; bp 700–702 GTA → CAA, Leu → Gln; and bp 781–783 TGG → TCG, Leu → Ser. To determine whether the sequencing differences were potentially arising from tissue-specific isoforms, 11β-HSD1 was amplified from a mouse hepatic cDNA library, using the same primers and PCR conditions as above. The same four differences from the NM_008288 sequence were obtained from multiple PCRs. Upon more exhaustive searches of GenBank™, a sequence for mouse hepatic 11β-HSD1 (S75207) was found that completely agreed with the present results from mouse adipocytic and hepatic libraries. We therefore conclude that this latter sequence is correct, and NM_008288 is incorrect. We have submitted the mouse adipocytic 11β-HSD1 sequence to GenBank™ (DQ089001).

**Generation and Affinity Purification of Polyclonal Anti-11β-HSD1 Antibodies—**

Human and mouse 11β-HSD1 sequences were compared, and an antigenic peptide (amino acids 22–39) from the mouse sequence that was highly conserved between both species was commercially synthesized (Invitrogen) and injected into two rabbits. The crude sera were tested against recombinant 11β-HSD1, and the bleed with the highest titer was affinity-purified as previously described (27) using the inoculating peptide immobilized on Affi-Gel-10 beads (Bio-Rad).

**Preparation of Adenooviral Constructs—**

For generation of shRNA constructs against 11β-HSD1, four 19-nucleotide sequences were designed to silence gene expression of 11β-HSD1 using the on-line shRNA Target Finder from Ambion. The candidate sequences chosen were (numbers correspond to base pairs in murine 11β-HSD1 sequence): UUUAAUGUCAG-GCGGGAA (bp 304–322), GAGUCAUGGAGGUCAACUU (bp 413–431), CUCUCUAACCCAGGCUCA (bp 601–619), and AUCUCUGGGAUAAUGACG (bp 679–687). The fourth

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sequence contained a mutation (underlined) caused by an error in the GenBank™ sequence used to design the constructs (NM_008288; see above) and was not subsequently used. Oligonucleotides containing the sense sequence, a hairpin loop region, and the antisense sequence were synthesized (IDT) and annealed to their respective complimentary sequences and subcloned into the pSIREN-shuttle vector (BD Biosciences, Palo Alto, CA). Successful insertion of the shRNA constructs into the vector was confirmed by sequencing. 293T cells in 12-well dishes were transiently transfected with 0.5 μg of pcDNA3.1 vector containing 11β-HSD1 and 0.5 μg of pSIREN-11β-HSD1 shRNA or pSIREN-control-shRNA using Lipofectamine PLUS (Invitrogen) according to the supplier’s instructions. After 24 h recovery, the cell lysates were prepared, and the 11β-HSD1 levels were analyzed by immunoblotting. Constructs 1, 2, and 3 caused a comparable reduction in co-transfected exogenous 11β-HSD1 expression, whereas construct 4 was largely without effect (data not shown). shRNA constructs 1 and 3 and a scrambled control construct were subcloned into the Adeno-X vector and purified as previously described (28). Both shRNA constructs induced a similar reduction in 11β-HSD1 levels in 3T3-L1 cells (data not shown), so construct 1 was used for all subsequent experiments. An adenoviral construct encoding full-length 11β-HSD1 was prepared, packaged, and purified as previously described (29).

Infection of 3T3-L1 Cells with Adenoviral Constructs—11β-HSD1 was overexpressed in 3T3-L1 adipocytes using adenovirally mediated gene transfer as described (29). For shRNA experiments, 3T3-L1 fibroblasts were plated on 12-well dishes and, upon reaching 60–80% confluence, were infected with varying titers of adenoviral shRNA constructs (10^5–10^6 particles/cell), using the same conditions as for the 11β-HSD1 adenoviral infections. After 2 days in the presence of adenovirus, the medium was removed, and the cells were subjected to the differentiation protocol as described above.

Preparation and Analysis of Cellular Lysates—The cells were washed three times with phosphate-buffered saline on ice. For immunoblotting experiments, the cells were scraped into homogenization buffer (50 mM Heps, pH 7.4, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 10% glycerol, 0.5% Triton X-100, and protease inhibitors added just before use). The lysates were centrifuged for 10 min, 10,000 × g, 4 °C, and the supernatants were transferred to new tubes. For immunoblots, the lysates were resolved on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Western blots were probed as described (30) with antibodies against anti-glycogen synthase and anti-adiponectin; (Chemicon, Temecula, CA); anti-protein phosphatase-1 (sc7482); anti-C/EBPα, anti-C/EBPβ, and anti-PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phosphotyrosine (Upstate Cell Signaling Solutions); anti-perilipin (Research Diagnostics Inc., Flanders, NJ); and anti-FLAG (Sigma). The blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Bio-Rad) and developed using ECL reagent (Amersham Biosciences).

GRE-Luciferase Assays—Transient transfection studies were carried out in 6-well plates using Lipofectamine Plus and a luciferase reporter construct cloned downstream of a GRE contained in a mouse mammary tumor virus promoter (generous gift of Dr. F. Wondisford, Johns Hopkins). Each 6-well plate was transfected with 10 μg of GRE-Luc, and 15–18 h after transfection, the cells were washed with phosphate-buffered saline, and treated with DMEM plus 10% FBS and the indicated additions. After 24 h of treatment, the cells were harvested by lysis for 15–20 min with shaking in 300 μl of buffer containing (25 mM glycilglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1% Triton X-100, and 2 mM dithiothreitol), 150 μl of cell lysate was then combined with an equal part assay buffer (25.6 mM glycilglycine, pH 7.6, 15 mM MgSO₄, 4 mM EGTA, 15 mM K₂HPO₄, 3 mM dithiothreitol, 6 mM ATP) and an equal part of solution containing 0.2 m luciferin (Invitrogen) dissolved in a modified lysis buffer (25 mM glycilglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA). Luciferase activity was subsequently measured using a Lumat LB 9507 Luminometer (Berthold Technologies, Oak Ridge, TN).

Statistical Analysis—Data comparisons were analyzed by Student’s t test. Analysis was performed using Microsoft Excel XP and was considered statistically significant at p < 0.05.

RESULTS

Dehydrocorticosterone Promotes 3T3-L1 Differentiation—The murine 3T3-L1 cell line is a widely used model for the study of adipogenesis. The inclusion of the synthetic glucocorticoid dexamethasone in the differentiation mixture is required for the optimal induction of adipogenesis. To test the ability of other murine glucocorticoids to promote 3T3-L1 differentiation, confluent 3T3-L1 fibroblasts were induced to differentiate by incubation with DMEM containing 10% FBS, 167 nM insulin, and 0.5 mM MIX. Additionally, 250 nM of the synthetic glucocorticoid dexamethasone, the active murine glucocorticoid corticosterone or the inactive glucocorticoid dehydrocorticosterone (DHC) was added. After completion of the standard protocol (see “Experimental Procedures”), the cells were analyzed by Oil Red O staining. The cells treated with medium lacking any glucocorticoids retained their fibroblast morphology, lacked detectable lipid droplet accumulation (Fig. 1), and exhibited very low levels of all adipocyte-specific proteins measured (supplemental Fig. S1). In contrast, inclusion of each of the three glucocorticoids in the differentiation protocol increased lipid accumulation (Fig. 1), induced the expression of several adipocytic proteins, and increased insulin signaling (supplemental Fig. S1). The superactive dexamethasone was more potent than corticosterone, which in turn was more potent than the inactive form DHC. Identical results were obtained with cortisone and cortisol, the inactive and active human glucocorticoids, respectively (data not shown). Cumulatively, these findings suggested that 3T3-L1 fibroblasts contain an enzymatic oxoreductase activity that catalyzes the activation of DHC or cortisone to drive adipogenesis.

11β-HSD1 Protein Levels Increase during 3T3-L1 Differentiation—We next analyzed the expression of 11β-HSD1 during 3T3-L1 differentiation. 11β-HSD1 protein levels were determined by immunoblotting using a novel affinity-purified, polyclonal peptide antibody that recognizes both murine and human isoforms of 11β-HSD1 (supplemental Fig. S2). 3T3-L1 adipocytes were differentiated by a standard 5-day protocol,
and on each day replicate wells were harvested. At the end of the time course, equal amounts of cellular lysates were separated by SDS-PAGE, and 11β-HSD1 levels were determined by immunoblotting. In parallel, the expression of several genes previously shown to be up-regulated at different stages of 3T3-L1 adipogenesis was analyzed. Differentiation of 3T3-L1 adipocytes initially resulted in the increased expression of C/EBPa and PPARγ (31), followed by perilipin, adiponectin, and glycogen synthase (Fig. 2A). In contrast, levels of protein phosphatase-1 were unchanged during differentiation (32) and used as a protein loading control. 11β-HSD1 expression also rose during 3T3-L1 differentiation (Fig. 2A), albeit toward the end of the protocol. Both the increased 11β-HSD1 expression and late induction are in agreement with previous results examining 11β-HSD1 mRNA expression during 3T3-L1 adipogenesis (33). Additionally, the timing of increased C/EBPa levels preceding 11β-HSD1 up-regulation suggested that this transcription factor may control 11β-HSD1 expression, as has been reported in liver cells (34). However, additional experiments would be required to prove a causal link.

To identify which agents in the differentiation mixture were responsible for driving 11β-HSD1 expression, confluent 3T3-L1 fibroblasts were treated for 3 days with DMEM containing 10% FBS and the various permutations of insulin, MIX, and dexamethasone (DHC). After completion of the differentiation protocol, the cell lysates were prepared and analyzed by anti-11β-HSD1 immunoblotting (Fig. 2B). Dexamethasone in combination with either MIX alone or MIX and insulin produced the strongest increase in 11β-HSD1 expression (Fig. 2B, lanes 8 and 12). DHC treatment produced similar results, although the induction of 11β-HSD1 protein was lower (Fig. 2B, lanes 9 and 11). Interestingly, the addition of insulin to the combination of MIX and DHC or dexamethasone reduced 11β-HSD1 expression (Fig. 2B, lanes 8 versus lanes 12 and 9 versus lane 11), suggesting an antagonistic role for insulin on 11β-HSD1 expression. In parallel, the lysates were also analyzed by anti-PPARγ immunoblotting. In agreement with previous results (35), the induction of PPARγ expression during differentiation was found to be completely dependent on the presence of glucocorticoid in the initial treatment condition (Fig. 2B, lanes 4, 5, and 7–12), whereas all of the combinations lacking the glucocorticoid had no change in levels of this important transcription factor.

shRNA-mediated Knockdown of 11β-HSD1 Blocks DHC-induced Differentiation of 3T3-L1 Cells—To more definitively assess the role of 11β-HSD1 in DHC-mediated 3T3-L1 differentiation, we employed RNA interference to specifically reduce 11β-HSD1 protein levels in 3T3-L1 fibroblasts. As detailed under “Experimental Procedures,” during PCR cloning of 11β-HSD1 from several murine adipocytic cDNA libraries, we consistently obtained four nonconservative substitutions compared with the widely referenced GenBank™ sequence NM_008288 for hepatic 11β-HSD1. To ensure that the differ-


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ences were not due to tissue-specific isoforms, we PCR-amplified 11β-HSD1 from a hepatic cDNA library and obtained the same four differences. A more exhaustive search of GenBank yielded a second sequence for hepatic 11β-HSD1 (S75207) that was in complete agreement with the adipocytic 11β-HSD1 sequence (DQ089001). The adipocytic construct was then cloned for transient expression in 293T cells and adenovirally mediated gene transfer in 3T3-L1 adipocytes.

Four potential shRNA constructs against murine 11β-HSD1 were identified using the Ambion website. As a control, a fifth scrambled sequence was also identified. As detailed under “Experimental Procedures,” the shRNA constructs were screened in transient transfection assays using 293T cells and exogenous 11β-HSD1. The two most efficacious sequences and a scrambled control were then prepared as adenoviral constructs and titered in fully differentiated 3T3-L1 adipocytes, because endogenous 11β-HSD1 levels were more readily detectable. Equivalent reduction in 11β-HSD1 protein expression in a dose-dependent manner was obtained with both shRNA constructs, whereas the scrambled construct was without effect (data not shown). Therefore, shRNA construct 1 was used for all subsequent experiments.

Replicate wells of 3T3-L1 fibroblasts were treated in the absence (mock) or presence of 5 × 10^5 particles/cell of 11β-HSD1 shRNA adenovirus and allowed to recover for 2 days. 3T3-L1 adipogenesis was initiated as above, using either 250 nM dexamethasone or DHC in the differentiation mixture. At the indicated time points (Fig. 3), the cell lysates were prepared and analyzed by immunoblotting. Several findings were apparent from the experiment: pretreatment of the fibroblasts with 11β-HSD1 shRNA largely blocked the initial induction of PPARγ and C/EBPα by DHC (Fig. 3, Day 3), as well as the later increases in adiponectin, perilipin, PPARγ, C/EBPα, and 11β-HSD1 expression (Fig. 3, Day 5). The residual expression of these proteins may be due to the high but incomplete adenoviral infection efficiency in these cells (28). In contrast, the ability of dexamethasone to increase expression of these proteins was unaffected, which is not surprising because this synthetic glucocorticoid is not dependent on 11β-HSD1-mediated activation. Finally, the 11β-HSD1 shRNA construct only partially reduced 11β-HSD1 levels in cells differentiated in the presence of dexamethasone. This finding most likely stems from the late induction of 11β-HSD1 expression during 3T3-L1 differentiation coupled with the single infection of the cells on Day 2 of differentiation, indicating that residual shRNA levels were relatively low when 11β-HSD1 mRNA expression began to rise. In contrast, because DHC was not activated on Day 0 in the shRNA-infected fibroblasts, 3T3-L1 differentiation was blunted at a very early stage, preventing the subsequent rise in endogenous 11β-HSD1 and all other proteins examined. Cumulatively, these results suggested that the conversion of DHC by endogenous 11β-HSD1 in 3T3-L1 fibroblasts was an initiating step in promoting adipogenesis by this glucocorticoid.

11β-HSD1 Overexpression in 3T3-L1 Cells Blocks Adipocyte Differentiation—Because 11β-HSD1 protein and mRNA expression increased during 3T3-L1 adipogenesis (Figs. 2 and 3 and Ref. 33), we next examined the effects of prematurely increasing 11β-HSD1 levels on the differentiation of 3T3-L1 fibroblasts. We employed a Tet-Off adenoviral system for the high efficiency infection of 3T3-L1 cells (29). Further, inclusion of the tetracycline analogue doxycycline (Dox) in the medium completely suppresses exogenous protein expression, which serves as a control for potential nonspecific effects arising from the viral infection. 3T3-L1 fibroblasts were infected with increasing titers of adenovirus encoding for FLAG epitope-tagged 11β-HSD1. Following 48 h of recovery in the absence or presence of 1 μg/ml Dox, the lysates were prepared and analyzed in parallel by anti-FLAG and anti-11β-HSD1 immunoblotting (supplemental Fig. S2).

We next examined the effects of 11β-HSD1 overexpression on 3T3-L1 differentiation by submaximal doses of DHC or corticosterone. The cells were infected with a titer of 11β-HSD1 adenovirus that increased exogenous enzyme expression in the preadipocytes to levels comparable with endogenous 11β-HSD1 expression in the fully differentiated 3T3-L1 adipocytes (Fig. 4, bottom panel). Two days later, the cells were incubated with the standard differentiation mixture containing increasing concentrations of either inactive DHC or active corticosterone (0–500 nm). As a control, 1 μg/ml Dox was added to half of the wells throughout the protocol to suppress exogenous 11β-HSD1 expression. After completion of differentiation, the lysates were prepared and analyzed by immunoblotting. Initially, 11β-HSD1 levels were determined. Inclusion of the FLAG epitope tag on exogenous 11β-HSD1 slightly reduced its electrophoretic mobility, enabling differentiation of exogenous versus endogenous 11β-HSD1 proteins by anti-11β-HSD1 immunoblotting (Fig. 4, ex-HSD versus en-HSD). In cells differentiated in the presence of Dox, which prevented exogenous 11β-HSD1 expression, both glucocorticoids increased endogenous 11β-HSD1 expression by the end of the protocol (Fig. 4, bottom right panels). In replicate plates, exclusion of Dox from
or 250 nM of DHC, corticosterone, or dexamethasone. The cells were then induced to differentiate as in Fig. 1, using increasing concentrations of either DHC (first and third panels) or corticosterone (second and fourth panels), in the continued absence and presence of Dox. On day 5, the lysates were then prepared and analyzed with either anti-glycogen synthase (GS) antibody or anti-11β-HSD1 antibody. Exogenous 11β-HSD1 (ex-HSD1) runs at a slightly lower electrophoretic mobility than endogenous 11β-HSD1 (en-HSD1) because of the inclusion of a FLAG epitope tag in the adenoviral construct. The results are representative of two independent experiments.

The previous results suggested that overexpressed 11β-HSD1 activity in 3T3-L1 fibroblasts catalyzed the inactivating, dehydrogenase reaction, thus blocking the adipogenic effects of both DHC and corticosterone. Direct measurement of 11β-HSD1 activity in 3T3-L1 fibroblasts completely inhibited differentiation by either glucocorticoid, as assessed by expression of glycogen synthase (Fig. 4, top panel), endogenous 11β-HSD1 (Fig. 4, bottom panel), adiponectin (supplemental Fig. S3), or Oil Red O staining (data not shown). These data indicated that increased 11β-HSD1 protein expression is temporally controlled during 3T3-L1 differentiation to prevent the inactivation of glucocorticoids necessary for initiation of the adipogenesis.

Finally, to measure the directionality of exogenous 11β-HSD1 activity in 3T3-L1 preadipocytes, cells were infected with 11β-HSD1 adenovirus, recovered 24 h with or without Dox, and then were transiently transfected with the GRE-luc construct. The cells were then stimulated overnight with each addition of GE reduced by over 60% the activation of the GRE-luc construct by DHC (Fig. 5B). In parallel, the effects of GE on 3T3-L1 differentiation were measured, and identical results were found; GE had no effect on dexamethasone- or corticosterone-induced induction of adiponectin or glycogen synthase expression, but GE completely blocked the induction of these proteins by DHC (Fig. 5C). Thus, the differential effects of GE on activation of the GRE-luc construct by glucocorticoids (Fig. 5B) were mirrored by corresponding effects on 3T3-L1 differentiation (Fig. 5C).
transgenic overexpression of 11β-HSD1 in the livers of mice results in mild insulin resistance, dyslipidemia, and hypertension (40). However, recent work has demonstrated that the localized activation of glucocorticoids by adipose tissue may play a pivotal role in the connection between glucocorticoids and the development of visceral obesity and insulin resistance. Adipocyte-specific overexpression of 11β-HSD1 in animals results in a significant increase in the localized concentration of active corticosterone in the fat pads (22). This finding correlated with the development of visceral obesity and the associated decrease in insulin sensitivity, resulting in hyperglycemia, and dyslipidemia. In addition, the transgenic overexpression of 11β-HSD2 under the control of the aP2 promoter to specifically inactivate glucocorticoids in adipose tissue resulted in mice that were resistant to weight gain on high fat diets and had increased glucose tolerance and insulin responsiveness (41). Thus, modulation of active glucocorticoid levels in fully differentiated adipocytes affects the metabolic profile in vivo.

The role of 11β-HSD1 in mediating glucocorticoid-driven adipocyte differentiation is less well understood, because use of the aP2 promoter in the transgenic animals would induce exogenous protein expression late during adipogenesis. There have been no reports concerning the effects of direct modulation of 11β-HSD1 protein levels in preadipocytes. In the present study, we utilized adenovirally mediated overexpression and shRNA delivery in 3T3-L1 cells to examine the effects of specific modulation of 11β-HSD1 protein levels on the initiation of adipogenesis by glucocorticoids.

We initially found that 3T3-L1 fibroblasts could be induced to differentiate by the five glucocorticoids tested: dexamethasone, corticosterone, cortisol, DHC, and cortisone. Of particular interest were the latter two compounds, because they are inactive glucocorticoids and presumably require an endogenous oxoreductase activity in 3T3-L1 fibroblasts to activate them prior to initiation of differentiation. This supposition was confirmed by the ability of the pharmacological 11β-HSD1 inhibitor GE to completely block DHC-mediated 3T3-L1 differentiation. In agreement, the inactive glucocorticoid cortisone has been reported to initiate human preadipocyte differentiation in vitro, and this effect was blocked by GE (24).

However, we were able to conclusively identify 11β-HSD1 as the enzymatic activity mediating the conversion of inactive glucocorticoids in 3T3-L1 fibroblasts, because RNA interference against 11β-HSD1 also completely blocked adipogenesis. In contrast, the inhibitor and shRNA reagents had no effect on the ability of active dexamethasone or corticosterone to drive differentiation.

Additionally, we found that the activation of DHC by endogenous 11β-HSD1 in 3T3-L1 cells must be an initiating step in the adipogenic program, because reduction of 11β-HSD1 levels by shRNA subsequently reduced the expression of all adipocyte-specific proteins measured, including the crucial transcription factors PPARγ and C/EBPβ that are induced early during adipogenesis. RNA interference against 11β-HSD1 had no significant effect on dexamethasone-induced differentiation, indicating that the complete ablation of DHC-driven adipogenesis was specific to reduction of 11β-HSD1 levels in fibro-
blasts, as opposed to nonspecific shRNA and/or adenoviral effects on these cells.

During the course of these studies, we determined that endogenous 11β-HSD1 protein levels markedly increased during 3T3-L1 differentiation, during the terminal stages of adipogenesis. Indeed, 11β-HSD1 was the last protein to be up-regulated of the seven adipocytic protein examined by immunoblotting. These results correspond to previous work that demonstrated the late induction of 11β-HSD1 mRNA levels during differentiation of 3T3-L1 and 3T3-F442A adipocyte cell lines (33). Thus, basal 11β-HSD1 activity present in 3T3-L1 fibroblasts is essential for the reduction and activation of DHC for this glucocorticoid to mediate 3T3-L1 adipogenesis and subsequently further increase 11β-HSD1 expression.

We next examined the effects of increasing 11β-HSD1 expression in fibroblasts on adipocyte differentiation. Surprisingly, we found that instead of facilitating DHC-induced 3T3-L1 differentiation, 11β-HSD1 overexpression completely blocked adipogenesis in the presence of DHC. Further, the ability of the active glucocorticoid corticosterone to drive differentiation was also suppressed. These results were not due to nonspecific effects of adenoviral infection, because inclusion of doxycycline in the medium completely suppressed exogenous 11β-HSD1 expression and restored the ability of these glucocorticoids to promote 3T3-L1 adipocyte differentiation. Additionally, the ability of the synthetic glucocorticoid dexamethasone, which is not a substrate for 11β-HSD1, to promote 3T3-L1 adipogenesis was not affected by 11β-HSD1 overexpression. Interestingly, the blockage of DHC- and cortisone-mediated 3T3-L1 differentiation was achieved by prematurely expressing 11β-HSD1 in 3T3-L1 fibroblasts to the levels of endogenous 11β-HSD1 found in fully differentiated 3T3-L1 adipocytes.

The inhibition of both DHC and corticosterone action by exogenous 11β-HSD1 suggested that the overexpression of the enzyme in 3T3-L1 fibroblasts resulted in the generation of a dominant, inactivating dehydrogenase activity. Using a GRE-luciferase reporter, we confirmed that GR activation by corticosterone or DHC was largely suppressed upon elevation of 11β-HSD1 levels. In contrast, the ability of dexamethasone, which is not a substrate for 11β-HSD1, to activate the GRE-luciferase construct or to drive 3T3-L1 adipogenesis was not significantly affected by 11β-HSD1 overexpression. During differentiation of stromal cells isolated from omental fat, 11β-HSD1 enzymatic activity switched from primarily an inactivating dehydrogenase activity to an activating oxoreductase activity (42). The directionality of 11β-HSD1 activity appeared to be determined by the availability of NADPH (36, 43) and the expression of the enzyme hexose-6-phosphate dehydrogenase (44). NADPH levels greatly increase during adipogenesis (45), potentially underlying the switch in 11β-HSD1 activity upon differentiation in stromal cells, as well as potentially in 3T3-L1 adipocytes. Thus, 11β-HSD1 activity during adipocyte differentiation appears to be regulated at both the level of expression and enzymatic directionality.

Cumulatively, these data suggest that 11β-HSD1 activity was tightly controlled during 3T3-L1 differentiation. The low levels of 11β-HSD1 in fibroblasts enabled the conversion of inactive glucocorticoids and initiation of adipocyte differentiation. Endogenous 11β-HSD1 levels increased late during adipogenesis, presumably when the differentiating cells have sufficient capacity to generate NADPH and preserve the oxoreductase activity of 11β-HSD1 and thus prevent glucocorticoid inactivation and suppression of the adipogenic program. These findings also suggest that transgenic overexpression of 11β-HSD1 in preadipocytes, rather than under control of the aP2 promoter, may result in inhibition of adipocyte formation and potentially protection from diet-induced obesity and insulin resistance. Recently, transgenic animals lacking hexose-6-phosphate dehydrogenase activity were described (44). Circulating corticosterone levels were markedly reduced because of conversion of 11β-HSD1 into a dehydrogenase enzyme, but the effects on adipose mass were not reported. Further work is clearly needed to address these issues and further understand the complex interplay between 11β-HSD1 expression and directionality of activity, localized glucocorticoid activation/inactivation, induction of adipogenesis, and development of insulin resistance.

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