Evidence That the 11 β-Hydroxysteroid Dehydrogenase (11 β-HSD1) Is Regulated by Pentose Pathway Flux

STUDIES IN RAT ADIPOCYTES AND MICROSONES

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11 β-hydroxysteroid dehydrogenase type 1 (11 β-HSD1) catalyzes the interconversion of biologically inactive 11 keto derivatives (cortisone, 11-dehydrocorticosterone) to active glucocorticoids (cortisol, corticosterone) in fat, liver, and other tissues. It is located in the intraluminal compartment of the endoplasmic reticulum. Inasmuch as an oxo-reductase requires NADPH, we reasoned that 11 β-HSD1 would be metabolically interconnected with the cytosolic glucocorticoid formation. To test this theory, 11 β-HSD1 activity and pentose pathway were simultaneously measured in isolated intact rodent adipocytes. Established inhibitors of NADPH production via the pentose pathway (dehydroandrostenedione or norepinephrine) inhibited 11 β-HSD1 oxo-reductase while decreasing cellular NADPH content. Conversely these compounds slightly augmented the reverse, or dehydrogenase, reaction of 11 β-HSD1. Importantly, using isolated intact microsomes, the inhibitors did not directly alter the tandem microsomal 11 β-HSD1 and hexose-6-phosphate dehydrogenase enzyme unit. Metabolites of 11 β-HSD1 (corticosterone or 11-dehydrocorticosterone) inhibited or increased pentose flux, respectively, demonstrating metabolic interconnectivity. Using isolated intact liver or fat microsomes, glucose-6 phosphate stimulated 11 β-HSD1 oxo-reductase, and this effect was blocked by selective inhibitors of glucose-6-phosphate transport. In summary, we have demonstrated a metabolic interconnection between pentose pathway and 11 β-HSD1 oxo-reductase activities that is dependent on cytosolic NADPH production. These observations link cytosolic carbohydrate flux with paracrine glucocorticoid formation. The clinical relevance of these findings may be germane to the regulation of paracrine glucocorticoid formation in disturbed nutritional states such as obesity.

The intracellular peri-receptor availability of glucocorticoids is not determined simply by their circulating concentrations and protein binding interactive kinetics. Ostensibly, the intracellular concentration of the active glucocorticoids (cortisol, corticosterone) is governed more so by 11 β-hydroxysteroid dehydrogenase type 1 (11 β-HSD1), a bidirectional enzyme that facilitates the equilibrium between the aforesaid active steroids and their biologically inactive 11-keto derivatives (cortisone, 11-dehydrocorticosterone) (1–3). 11 β-HSD1 is ubiquitous (4), located in microsomes (5), and has a low substrate affinity (6) ($K_m$ in $\mu M$) involving pyridine nucleotide cofactors, with NADPH having a greater affinity than NADH (6, 7). Recently, 11 β-HSD1 has garnered attention as a potential participant in the pathobiology of obesity, insulin resistance, and type II diabetes (2, 3, 8, 9). Specifically, the dysregulation of 11 β-HSD1 in particular tissues may augment intracellular active glucocorticoid concentrations. In addition, it is certainly plausible that in human obesity, although normal circulating blood cortisol levels are found, intracellular cortisol concentrations may be elevated, conceivably because an alteration in the NADPH/NADP ratio may foster 11 β-HSD1 reductase over dehydrogenase. Given the well recognized regulation by glucocorticoids of numerous homeostatic and metabolic processes of intermediary metabolism, the role of 11 β-HSD1 in obesity is under question (2, 8, 10–19). In addition to modulating several key intermediary enzymes, cortisol is pivotal in adipogenesis by promoting the differentiation of stromal cells (preadipocytes to adipocytes) (9–11, 20–22).

In vitro studies in intact cells from liver, adipose, and other tissues have consistently found the directionality of 11 β-HSD1 to be predominantly 11β oxo-reduction of inactive cortisone to cortisol (1, 9, 23–26). Interestingly, in human stromal cells from the omentum, upon differentiation to mature adipocytes, the 11 β-HSD1 activity converts from a dehydrogenase to an oxo-reductase (9). Antithetically, in disrupted cellular homogenates, the direction is reversed, where the enzyme functions preferentially as a dehydrogenase (6, 23, 26). The grounds for this paradox are poorly understood, but obviously substrate concentrations, ionic milieu, pH, intracellular location, and membrane binding may explain the kinetic discordance between intact cells versus homogenates (where reactions are often measured under artificial $V_{max}$ conditions). All these factors notwithstanding, the most plausible determinate of the direction of 11 β-HSD1 is the mass action effect of pyridine nucleotide cofactors. To date, no studies using intact cells have attempted to correlate ongoing in situ pentose pathway flux, which is recognized as the major intracellular producer of NADPH, with the simultaneous measurement of 11 β-HSD1 activity. Furthermore, to what extent the hormonal and metabolic manipulation of adipocyte pentose pathway (PP) may impact in situ microsomal 11 β-HSD1 activity has yet to be explored.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media and ingredients were from Invitrogen, general chemicals from Sigma, radioisotopes ([1-14C]glucose and [6,14C]glucose) from Amersham Biosciences, and 11-dehydrocorticosterone (11-DHC) from Steraloids, Inc., Newport, RI.

Rat Adipocyte Preparation—Isolated adipocytes were prepared from gonadal depots by standard collagenase digestion methods (27).
Adipocyte 11-β-Hydroxysteroid Dehydrogenase

Rodent Fat Cell Incubation Conditions—Isolated fat cells from 4–6 rats were pooled and distributed for metabolic studies into incubation tubes (three sets of duplicated tubes; set 1, measure PP flux with [1-14C] glucose, set 2, measure [6-14C] glucose oxidation (as below), and set 3, measure 11 β-HSD1 activity). All cell groups were distributed, handled, and analyzed in parallel under identical incubation conditions (37 °C). Isolated rat fat cells were incubated in 1–2-ml polypropylene tubes (set in 20-ml vials with Teflon/silicone membrane caps for CO2 collection, PP as below).

Rat Microsome Preparation—Liver microsomes were prepared according to the methods of Raucy and Lasker (28). Adipocyte microsomes were prepared from the pooled fat cells of three to four rats by similar methods (29).

11 β-HSD1 Activity—The o xo-reductase direction of 11 β-HSD1 in which dehydrocorticosterone (11-DHC) is converted to corticosterone (C) was measured in intact cells and microsomes. Isolated rodent fat cells (0.25 ml) were incubated in 0.1 mιl glucose Krebs buffer at 37 °C (final assay volume 0.4 ml, final cell concentration 5–6 × 10^5 ml or with microsomes 50 μg of protein/ml). The reaction was initiated with 250–1000 nιl 11-DHC (0.15 μl), incubated for 45–90 min, and terminated by freezing on dry ice. The appearance of C in the assay medium (conditioned medium) of isolated intact cells was determined by 3H radioimmuno assay of corticosterone (MP Biomedicals, Irvine, CA). Baseline studies examined the effect of substrate concentration (0–2 μM 11-DHC), cell number (0–5–6 × 10^6 fat cells/ml) and time (0–120 min) on corticosterone production to verify consistent linear rates of 11 β-HSD1 activity throughout the assay. The dehydrogenase reaction of 11 β-HSD1 (C → 11-DHC) was measured in intact cells under identical assay conditions by the disappearance of [3H] corticosterone (as above) at a final concentration of non-labeled substrate of 100 nM.

Pentose Pathway Flux—The conversion of [1-14C]glucose to 14CO2 followed previously described methods for 14CO2 collection and quantification (30–32). To correct PP flux for minor 14CO2 release from [1-14C]glucose generated through the glycolytic/citric acid cycle (33–35), the release of 14CO2 from [6-14C] was also determined in parallel experiments. The incubation medium was a 0.1-ml dissociated glucose Krebs buffer, pH 7.4. Metabolic reactions were started by the addition of assay mixture containing substrate (11-14C]glucose or [6-14C]glucose). Methods for this latter assay were identical to the PP assay with the exception that [6-14C]glucose replaces [1-14C]glucose.

Glucose Dehydrogenase—Isolated microsomes were tested for glucose dehydrogenase activity in the presence and absence of CHAPSO (a zwitterionic, non-denaturing detergent) as an index for microsomal membrane integrity. To mimic our experimental conditions, microsomes were first preincubated for 90 min at 37 °C in Krebs buffer. Next, they were washed once with 0.15 mιl Tris-HCL, pH 8.0 and incubated for 30 min at 4 °C with 0.8% CHAPSO. Glucose dehydrogenase activity was then monitored over 180 min at 25 °C in this same Tris buffer with 50 mιl Na2SO4 (36, 37).

NADPH Measurement—NADPH was measured by an ultraviolet radioisotopic assay (38).

Statistical Analysis—Data are expressed as a % control (no modifier), mean ± S.D. (n = 3–4). In each case, "n" refers to an individual experiment performed on 1 day with a fresh batch of cells or microsomes. Statistical differences were determined by paired t-test.

RESULTS

Baseline Characteristics of 11 β-HSD1 Oxo-reductase Assay—The 11 β-HSD1 oxo-reductase assay was measured in both isolated rat adipocytes and liver or fat microsomes. The reaction was linear over time (0–90 min), and its apparent K_m was 0.25 μM 11-DHC. The baseline rate of corticosterone formation from 11-DHC was 32.9 ± 4.8 ng of corticosterone formed/h/10^5 in adipocytes and 213.5 ± 62.1 ng of corticosterone/mg protein/h liver microsomes (with 1 μM glucose-6-phosphate (G6P)).

Dihydroepiandrostenedione (DHEA) and Norepinephrine (NE) Inhibit Both Pentose Pathway and 11 β-HSD1 Activity—Pentose pathway and 11 β-HSD1 activities were simultaneously measured and isolated adipocytes without and with 100 μM DHEA (Fig. 1, A and B) or 1 μM NE (Fig. 1, C and D). DHEA inhibited both the PP and oxo-reductase activities by 56–60% (p < 0.01) over the 90-min assay interval. Similarly, the adrenergic agent, NE, attenuated adipocyte pentose pathway (Fig. 1C) and 11 β-HSD1 oxo-reductase (Fig. 1D) activities by 54–60%. Importantly, reaction rates were linear and cell counts as well as lactate dehydrogenase release (cellular integrity) were stable (>90% baseline) with or without either inhibitor. It is noteworthy that DHEA did not directly affect 11 β-HSD1 enzyme activities in ruptured or homogenized fat cells. To determine whether the inhibition of these agents directly affected hexose-6-phosphate dehydrogenase (H6PD), which is the microsomal counterpart of cytosolic G6PD, isolated liver microsomes were incubated with and without DHEA or NE and 11 β-HSD1 oxo-reductase measured. As shown in Fig. 2, neither agent altered microsomal 11 β-HSD1 oxo-reductase. To determine whether the inhibitory actions of DHEA and NE on 11 β-HSD1 (as shown in Fig. 1, B and D) were enzyme specific, the reverse reaction (11- dihydrocorticosterone formation) was measured with and without these compounds. If these agents inhibit 11 β-HSD1 oxo-reductase by curtailing the supply of NADPH, then, conversely, they should stimulate the dehydrogenase direction. Both agents stimulated the dehydrogenase reaction by 2.5–3-fold (Fig. 3), although the effect of NE was not statistically significant.
Metabolites of the 11β-HSD1 Enzyme Reaction Regulate Pentose Pathway Activity in Isolated Fat Cells—If PP and 11β-HSD1 are metabolically linked, then the substrate/products of the 11β-HSD1 reaction might in turn affect PP flux. To examine this possibility, fat cells were preincubated with either 10 μM corticosterone or 11-DHC and then pentose pathway flux measured over 45 min. As predicted from Fig. 10, corticosterone, which would promote dehydrogenase flux (stimulates 11-DHC formation and an increased NAPDH/NADP ratio), inhibited PP by 30% whereas 11-DHC (stimulates corticosterone formation and, hence, reduces the NAPDH/NADP ratio) stimulated PP by 23% (Fig. 4). The basal dehydrogenase rate was 0.9 ± 0.06 ng/h/10⁶ cells.

G6P stimulates 11β-HSD1 Oxo-reductase in Microsomes—If the microsomal membrane transports G6P, then generation of this metabolite by cytosolic PP would lead to enhanced 11β-HSD1 activity. To test this hypothesis, isolated liver microsomes were incubated with increasing concentrations of G6P. G6P stimulated liver microsomal 11β-HSD1 reductase activity (Fig. 5). However, this stimulating effect was less so in isolated adipocyte microsomal membranes with 1, 5, and 10 mM G6P inasmuch as this compound activated 11β-HSD1 activity by 19 ± 3, 28 ± 3, and 60 ± 7% over basal (no G6P). These results were significant at p < 0.025, p < 0.01, and p < 0.01, respectively.

Microsomal Membrane Intactness Demonstrated by the Latency of Glucose Dehydrogenase Activity—The intactness of our experimental microsome preparation was tested by measuring glucose dehydrogenase activity following preincubation with and without the detergent membrane solubilizer (0.8% CHAPSO for 30 min at 4°C). Glucose dehydrogenase is an NADP+–requiring intraluminal microsomal enzyme whose activity is largely undetectable in intact microsomes (36). The results show that untreated microsomes were largely intact (no detectable glucose dehydrogenase activity over a 180-min incubation period) (Fig. 6).

G6P Translocase Inhibitors Block Microsomal 11β-HSD1 Activity—To determine whether microsomal 11β-HSD1 activity was dependent on transport-dependent G6P uptake/metabolism we examined 11β-HSD1 activity in whole microsomes with and without specific inhibitors of G6P uptake (chlorogenic acid or phlorizin). As an additional control for the specificity of G6P transport/metabolism, we tested the effect of an alternative sugar phosphate (galactose-1-phosphate). As shown using liver microsomes (Fig. 7), chlorogenic acid caused a 96% decrease in G6P-stimulated 11β-HSD1 activity, whereas Gal-1-P was ineffective. Similarly, using isolated fat microsomes, 11β-HSD1 activity in the presence of G6P was decreased to 52.6% ± 10.7% of basal (100%) with chlorogenic acid (p < 0.05 versus basal) and to 41.0 ± 6.9% of basal with phlorizin (p < 0.05 versus basal).

DHEA and Norepinephrine Reduce Adipocyte NADPH Content—If DHEA and norepinephrine regulate 11β-HSD1 oxo-reductase indirectly by inhibiting PP, then these inhibitors should, consequently, diminish adipocyte NADPH content. Therefore, the cellular content of this pyridine nucleotide phosphate was measured after 45 min of preincubation with either 100 μM DHEA or 1 μM NE (Fig. 8). The respec-
Adipocyte 11β-Hydroxysteroid Dehydrogenase

**FIGURE 6.** Effect of CHAPSO detergent on the latency of liver microsomal glucose dehydrogenase activity. Isolated liver microsomes (250 μg/ml) were preincubated with Krebs buffer for 90 min at 37 °C and then treated without or with CHAPSO before the glucose dehydrogenase assay was performed (see "Experimental Procedures"). Glucose dehydrogenase activity is represented as A₄₂₀ over time (mean ± S.D., n = 3). Of note, there was no detectable glucose dehydrogenase activity in the absence of glucose or microsomes. Differences (control versus CHAPSO) were statistically significant by paired t-test at all time points (p < 0.001).

**FIGURE 7.** Effect of the G6P translocase inhibitor, chlorogenic acid, on liver microsomal 11β-HSD1 oxo-reductase activity. Liver microsomes (50 μg/ml) were preincubated for 60 min with and without 0.5 mM chlorogenic acid and either 0.5 mM G6P or 1 mM galactose-1-phosphate. 11β-HSD1 oxo-reductase activity was then measured over 45 min. One representative experiment is shown.

**FIGURE 8.** Effect of DHEA and NE on adipocyte NADPH content. Fat cells were incubated with 100 μM DHEA or 1 μM NE, and then total cellular NADPH content was measured in washed fat cells by a radioisotopic assay as shown under "Experimental Procedures." Results are from four independent experiments and are presented as mean ± S.D., relative to control (no additions) with statistical differences by paired t-test (shown). The NADPH content of the control adipocytes was 8.6 ± 2.2 pmol/10⁶ cells.

**FIGURE 9.** Effect of pyridine nucleotides on liver microsomal 11β-HSD1 oxo-reductase activity. Rat liver microsomes (50 μg/ml) were preincubated for 60 min at 37 °C with the pyridine nucleotides noted (each at final concentration of 1 mM) and then 11β-HSD1 oxo-reductase activity measured over 45 min as shown under "Experimental Procedures." Results are expressed relative to basal (no modifier, no G6P) for three separate experiments (mean ± S.D.). The control rate was 239 ± 70 ng of corticosterone/h/mg protein. Statistical differences are by paired t-test.

NADPH caused significant (7-fold) increase in the activity of this intraluminal enzyme, whereas NADH had a lesser activating effect (166 ± 15% of basal). On the other hand, NADP was inhibitory (74 ± 6% basal) (Fig. 9). These data, along with the evidence for membrane intactness (Fig. 6), support that the microsomal membrane is not completely impermeable to NADPH over a prolonged incubation.

**DISCUSSION**

Numerous studies have commented on the enigmatic directionality of microsomal 11β-HSD1, seemingly dependent on the enzyme status: in intact cells oxo-reductase predominates, but when the enzyme is studied under cell-free conditions, the dehydrogenase direction prevails.

Indirect evidence for the necessity of a renewable source of NADPH to sustain microsomal 11β-HSD-1 reductase is manifest in patients with cortisol reductase deficiency (39). The latter results in inadequate regeneration of cortisol, subsequent overstimulation of adrenocorticotropic release, and a chronic mild androgen excess that promotes the polycystic ovarian phenotype. These patients have triallelic digenic mutations, ostensibly dosage dependent, involving not only 11β-HSD1 but also H6PD, the microsomal counterpart of cytosolic glucose-6-phosphate dehydrogenase. Notably, H6PD is a bifunctional enzyme that also has 6-phosphogluconolactonase catalytic activity; hence, it contains the oxidative portion of the pentose pathway (40). Not only are these patients deficient in 11β-HSD1 but they have a concomitant decrease in H6PD activity; therefore, impaired micrososomal NADPH production nullifies any residual reductase activity. Another recent article buttresses the importance of luminal H6PD in the regulation of 11β-HSD1. In human embryonic kidney 293 cells, in which endogenous expression of theses two enzymes is normally scant, co-expression studies revealed co-localization of these two enzymes in the ER. In addition, enhanced expression of H6PD stimulated 11β-HSD1 reductase activity (41).

In our fat cell experiments, after the targeted inhibition of G6P dehydrogenase with the non-competitive inhibitor DHEA, the resultant 56% reduction in adipocyte pentose flux (Fig. 1A) produced a significant attenuation of the oxo-reductase activity of 11β-HSD1 (Fig. 1B). Cellular NADPH content likewise declined (Fig. 8). In addition, if there is a kinetic linkage between pentose pathway flux ⇨ NADPH ⇨ 11β-HSD1, this reduction in NADPH by DHEA should promote the dehydrogenase direction for 11β-HSD-1. Indeed, DHEA caused a significant 187% increase (Fig. 3). Further experimental corroboration for this link-
Adipocyte 11β-Hydroxysteroid Dehydrogenase

FIGURE 10. Schematic illustration of proposed metabolic interconnection between cytosolic pentose pathway and 11β-HSD1 in the endoplasmic reticulum (ER). Glucose enters the cell via a plasma membrane transporter (T) and is phosphorylated to glucose-6-phosphate (G-6-phosphate). This hexose phosphate is metabolized via glycolysis or the cytosolic oxidative pentose pathway (wherein NADP is converted to NADPH), or it may also be transported via its specific transporter (T) directly into the ER. NADPH produced in the cytosol accesses the ER via an uncertain mechanism, graphically depicted here as a pore for the sake of simplicity. This cytosolic-derived NADPH is then utilized by the intraluminal 11β-HSD1 oxo-reductase to convert the biologically inert 11-dehydrocorticosterone (or cortisone in humans) to biologically active corticosterone (or cortisol in humans). Also depicted is hexose-6-phosphate dehydrogenase (H6PD), residing proximate to 11β-HSD1, which can locally produce NADPH within the ER space from transported G-6-phosphate.

age was borne out in the NE studies. Prior reports (34) have confirmed that this hormone potently attenuates PP flux in adipocytes, most likely by stimulating glycolysis. In our fat cell studies, norepinephrine caused a 54% decrease in PP flux (Fig. 1C), 47% reduction in NADPH content (Fig. 8), and a concomitant 40% reduction in cytosolic oxidative pentose pathway (wherein NADP is converted to NADPH), or it may also be transported via its specific transporter (T) directly into the ER. NADPH produced in the cytosol accesses the ER via an uncertain mechanism, graphically depicted here as a pore for the sake of simplicity. This cytosolic-derived NADPH is then utilized by the intraluminal 11β-HSD1 oxo-reductase to convert the biologically inert 11-dehydrocorticosterone (or cortisone in humans) to biologically active corticosterone (or cortisol in humans). Also depicted is hexose-6-phosphate dehydrogenase (H6PD), residing proximate to 11β-HSD1, which can locally produce NADPH within the ER space from transported G-6-phosphate.

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Adipocyte 11β-Hydroxysteroid Dehydrogenase

another study it was found that NADP can slowly enter liver microsomes (56). On the other hand, a recent study demonstrating cooperativity between 11β-HSD1 and H6PD in rat liver microsomes found evidence of meager, if any, NADP trans-membrane influx (57). In this report, the calculated ER latency of H6PD was 90%.

As to the tightness of the ER membrane barrier, this issue is beclouded by manifold methodological approaches including: use of permeabilized cells, differences in microsomal preparations and cell types, salt concentrations, duration of exposure to study compound, etc. To the point, a detailed study (58) in HeLa cells disclosed that the influx across the ER membrane of small (molecular mass 557 Da), non-physiologic, polar reagents is surprisingly brisk. This report did not study compounds of slightly higher molecular mass such as NADP. Although the ER membrane was quite leaky, both the plasma and lysosomal membranes were impermeable to these same compounds (58). Indeed, the ER translocon pore is estimated to be 40 – 60 Å, allegedly the largest diameter hole in a relatively impermeable eukaryotic membrane (59). One difference in some of the aforementioned studies and our series of experiments was the duration of time that the microsomes were exposed to the pyridine nucleotides and the duration of time the fat cells were exposed to the DHEA and NE. For example, in native microsomes, G6P stimulated 11β-HSD1 (by providing luminal NADPH) but NADPH per se failed to do so over a 5-min time course (57). In our intact microsomal studies 60 min of preincubation with NADPH (1 mM) activated 11β-HSD1 during the 45-min assay interval by >7-fold (Fig. 9). The reason for the discordance with our observations is unknown, but the incubation time may be germane. All this notwithstanding, in whole cells the manipulation of cytosolic PP activity with compounds that had no effect on H6PD or 11β-HSD1 in isolated microsomes caused a significant attenuation in 11β-HSD1 (Fig. 1, B and D).

In our experiments in intact cells the measurement of pentose pathway flux represents both the cytosolic and microsomal (40, 60) oxidative enzymes. Radiolabeled glucose-6-phosphate is generated intracellularly by hexokinase, and this compound can be taken up by microsomes. Consequently, our metabolic studies cannot discern to what extent the oxidative pentose flux is transpiring in the cytosol versus ER. However, quantitatively, as reported in rat tissue, the pentose pathway flux represents both the cytosolic and microsomal (40, 60).

In conclusion, pentose pathway activity modifies 11β-HSD1 activity in adipocytes, thereby linking carbohydrate metabolism to glucocorticoid production. This premise is supported by the following observations. (i) Curtailing adipocyte pentose flux with DHEA or NE reduces 11β-HSD1 oxo-reductase and augments dehydrogenase activity. Neither of these compounds had a direct effect on microsomal 11β-HSD1. (ii) The addition to fat cells of 11β-HSD1 substrates, such as corticosterone (which generates NADPH) or 11-dehydrocorticosterone (which generates NADP), had the predicted directional effect on pentose pathway. (iii) NADPH has some access to the microsomal membrane as evidenced by the marked stimulation of 11β-HSD1 oxo-reductase induced by this compound in isolated microsomes. This study suggests a constitutive metabolic interplay between intermediary carbohydrate metabolism and glucocorticoid production.

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Adipocyte 11 β-Hydroxysteroid Dehydrogenase

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