5α-Reduced Glucocorticoids, Novel Endogenous Activators of the Glucocorticoid Receptor*  

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Metabolism of glucocorticoids to A-ring-reduced dihydro- and tetrahydro-derivatives by means of hepatic 5α- and 5β-reductases has long been regarded as a pathway of irreversible inactivation. However, 5α-reduced metabolites of other steroids, e.g. testosterone and aldosterone, have significant biological activity. We investigated whether 5α-reduced metabolites of corticosterone are glucocorticoid receptor (GR) agonists. Corticosterone, 5α-tetrahydrocorticosterone (5αTHB), and 5α-dihydrocorticosterone (5αDHB) were similarly effective in displacing tritiated dexamethasone from binding sites in hepatocytes, whereas 5β-reduced metabolites were less effective in binding. 5αTHB had glucocorticoid receptor agonist effects in vitro and in vivo. After transient co-transfection of HGR and a murine mammary tumor virus-luciferase reporter into HeLa cells, 5αTHB was active to a comparable extent as corticosterone (28-fold versus 37-fold induction, respectively, at 1 μM) and additive to the effect of corticosterone. 5β-Reduced metabolites did not activate GR. In H4IE hepatoma cells, both 5αTHB and corticosterone induced mRNA expression of tyrosine aminotransferase and phosphoenolpyruvate carboxykinase (6.0- versus 10.1-fold and 3.5- versus 3.9-fold at 1 μM, respectively), an effect that was inhibited by RU486. To assess in vivo glucocorticoid activity, suppression of plasma ACTH was demonstrated in adrenalectomized rats after intraperitoneal administration of vehicle (ACTH trough 80.2 pg/ml, corticosterone (5 mg/kg; 22 pg/ml, 0.001) or 5αTHB (5 mg/kg; 51.3 pg/ml, p < 0.005). Similar endogenous concentrations of corticosterone and 5αTHB were detected in rat liver homogenates by gas chromatography mass spectrometry. We conclude that 5α-reduced glucocorticoids bind to and activate GR. Transcription of glucocorticoid-regulated genes in tissues that express 5α-reductases will thus be influenced by intracellular levels of both corticosterone and its 5α-reduced metabolites.

The rate-limiting step in glucocorticoid metabolism is the reduction of the Δ\(^4\) double bond in the A-ring of the steroid structure. This reaction is catalyzed by either 5α- or 5β-reductase. The resulting dihydro-metabolites are then reduced further by 3α-hydroxysteroid dehydrogenases to form tetrahydro-metabolites (Fig. 1). Two genes encoding 5α-reductase type 1 and type 2 isoforms have been identified (1). 5α-Reductase type 1 predominates in androgen-independent tissues such as the skin, liver, and adipose tissue, whereas 5α-reductase type 2 is the predominant isozyme in reproductive tissues (2).

5α-Reduction of glucocorticoids has been accepted as a pathway of irreversible inactivation (3). However, 5α-reduced metabolites of other steroids may be potent agonists at the same receptor as their parent hormone. 5α-Reductases catalyze the conversion of testosterone to the more potent androgen receptor agonist dihydrotestosterone (4). Both 5α-dihydroaldosterone and 3α,5α-tetrahydroaldosterone possess mineralocorticoid activity in rat kidney (5). The progesterone metabolites, 5α-dihydropregosterone and allopregnanolone may act as neurosteroids, and allopregnanolone binds to the non-nuclear γ-aminobutyric acid, type A receptor (6). An early study by Carlstedt-Duke et al. (3) concluded that 5α-dihydrocortisosterone was practically devoid of glucocorticoid activity and competed for dexamethasone binding sites much less efficiently than corticosterone. However, Baxter and Tomkins (7) found in rat hepatoma cells that 5α-dihydrocortisol induced tyrosine aminotransferase to a third of the level induced by cortisol. 5α-Reduced glucocorticoids may also regulate the glucocorticoid receptors as their parent hormone. 5α-reductases catalyze the conversion of testosterone to the more potent androgen receptor agonist dihydrotestosterone (4). Both 5α-dihydroaldosterone and 3α,5α-tetrahydroaldosterone possess mineralocorticoid activity in rat kidney (5). The progesterone metabolites, 5α-dihydropregosterone and allopregnanolone may act as neurosteroids, and allopregnanolone binds to the non-nuclear γ-aminobutyric acid, type A receptor (6). An early study by Carlstedt-Duke et al. (3) concluded that 5α-dihydrocortisosterone was practically devoid of glucocorticoid activity and competed for dexamethasone binding sites much less efficiently than corticosterone. However, Baxter and Tomkins (7) found in rat hepatoma cells that 5α-dihydrocortisol induced tyrosine aminotransferase to a third of the level induced by cortisol. 5α-Reduced glucocorticoids may also regulate the glucocorticoid receptors as their parent hormone. 5α-reductases catalyze the conversion of testosterone to the more potent androgen receptor agonist dihydrotestosterone (4). Both 5α-dihydroaldosterone and 3α,5α-tetrahydroaldosterone possess mineralocorticoid activity in rat kidney (5). The progesterone metabolites, 5α-dihydropregosterone and allopregnanolone may act as neurosteroids, and allopregnanolone binds to the non-nuclear γ-aminobutyric acid, type A receptor (6). An early study by Carlstedt-Duke et al. (3) concluded that 5α-dihydrocortisosterone was practically devoid of glucocorticoid activity and competed for dexamethasone binding sites much less efficiently than corticosterone. However, Baxter and Tomkins (7) found in rat hepatoma cells that 5α-dihydrocortisol induced tyrosine aminotransferase to a third of the level induced by cortisol.

EXPERIMENTAL PROCEDURES

Glucocorticoid Binding in Rat Hepatocytes—Male lean Zucker rats (6–8 weeks old, Harlan Olac Ltd, Bicester, UK; n = 6 per group) were anesthetized with sagatal (0.3 ml), the portal veins were cannulated, and livers were perfused in situ at a flow rate of 25 ml/min with a calcium-free buffer followed by a solution of collagenase type IV (Sigma) in Krebs buffer (10). After tissue dissociation, hepatocytes were isolated by filtration through a nylon mesh (aperture = 0.125 mm). Hepatocytes were separated from dead cells by repeated low speed centrifugation (500 × g, 2 min), resuspended in hepatocyte incubation buffer (120 mM Krebs buffer with NaCl, 5 mM glucose, 10 mM lactate, 2 mM glutamine, 1 mM pyruvate, and bovine serum albumin (2 g/100 ml buffer)) and and

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1 The abbreviations used are: GR, glucocorticoid receptors; 5αTHB, 5α-tetrahydrocorticosterone; 5αDHB, 5α-dihydrocorticosterone; 5βDHB, 5β-dihydrocorticosterone; 5βTHB, 5β-tetrahydrocorticosterone; TAT, tyrosine aminotransferase; GCMS, gas chromatography mass spectrometry; MMTV, murine mammary tumor virus; ACTH, adrenocorticotropic hormone; LAGS, low affinity glucocorticoid binding sites; GRE, glucocorticoid response element.
insulated at 37 °C for 30 min. Cell viability was determined by trypan blue exclusion under a light microscope.

For competitive binding experiments, duplicate incubations of hepatocytes (2 × 10^6/ml) were carried out at 4 °C overnight with ^3H_1_ dexamethasone (1.5 nM final concentration in molybdate buffer (25 μl, 0.1 M, pH 7.2) and non-radioactive competitor steroid (Steraloids, Newport, RI) dissolved in molybdate buffer (25 μl, final concentration from 0.63 nM to 200 μM). The competitors were dexamethasone, corticosterone, 5α-tetrahydrocorticosterone (5αDHB), 5β-dihydrocorticosterone (5βDHB), and 5β-tetrahydrocorticosterone (5βTHB).

Results were expressed as percentage of tritiated dexamethasone bound in the absence of competitor.

**Glucocorticoid Receptor Activation in Cell Lines**—Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 200 μM L-glutamine and grown at 37 °C and 5% CO₂. Transient transfections were carried out using the calcium phosphate co-precipitation method. HeLa cells were seeded at 3 × 10⁶/60-mm plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After overnight incubation, medium was replaced and cells were transfected with a total of 1 μg pCMV-GRα (Pharmacia, St. Albans, UK; internal control), 5 μg pLTR-Luc (11), 1 μg pRSGrα (11), and pGEM-3 (Promega, Southampton, UK). After over-night incubation, medium was replaced and steroid was added for 16 h before harvesting and lysis of cells. Luciferase and β-galactosidase activities were measured in cell lysates as described previously (12). β-Galactosidase activity was assayed using a Tropix Gallyas Light Plus kit. All transfections were carried out in triplicate and the mean ratio of luciferase/β-galactosidase activities was calculated.

Induction of tyrosine aminotransferase (TAT) and phospho-enolpyruvate carboxykinase (PEPCK) mRNA were assessed in H4IE cells treated overnight with corticosterone, 5αTHB (1 μM in ethanol at <0.1% final volume) or vehicle and/or RU486 (1 μM; a GR antagonist). Total RNA was extracted by using TRIzol reagent (Invitrogen), and Northern analysis was carried out by using a Fuji FLA2000 PhosphorImager analyzer.

**Metabolic Transformation of Steroids**—Medium was retained at 4 °C, allowed to return to 37 °C, and the dried residue was dissolved in methanol (0.5 ml) followed by water (5 ml). Medium was loaded on to the column, and the eluant was discarded. The column was washed with water (5 ml) and steroids were eluted in methanol (2 ml). The eluant was dried under nitrogen at 60 °C and resuspended in ethyl acetate (2 ml) and water (200 μl). The organic layer was separated and dried before derivatization to form methoxime-trimethylsilyl derivatives as described (16). Gas chromatographic mass spectrometric analysis was performed in electron impact mode (70 eV) using a Polaris Q ion-trap gas chromatography mass spectrometer (GCMS; Thermofinnigan, UK) (17) and intra-assay coefficients of variation were <10%.

**In Vivo Glucocorticoid Activity and Suppression of ACTH**—Adult lean male Zucker rats (6–8 weeks old, Harlan Olac Ltd, n = 6 per group) were fed ad libitum and maintained on a 12-h dark/light cycle. Rats were bilaterally adrenalectomized under halothane anesthesia and given 0.9% saline drinking water for 1 week before steroid treatment. Corticosterone, 5αDHB (both 5 mg/kg body weight) or vehicle (saline with 10% ethanol and 10% dimethyl sulfoxide) was injected intraperitoneally at 0900 h. Blood samples were taken at 0, 60, and 120 min after injection, and plasma was stored at −80 °C.

**Plasma ACTH concentrations** were determined by radioimmunoassay (Eurodiagnostica, Arnhem, The Netherlands). The inter- and intra-assay coefficients of variation were <4%. Plasma corticosterone levels were measured using an in-house radioimmunoassay (18). The inter- and intra-assay coefficients of variation were <10%.

**Tissue Concentrations of Steroids**—Livers (−500 mg) from adult lean male Zucker rats (6–8 weeks old, n = 3) were homogenized in ice-cold potassium phosphate buffer (pH 7, 5 vol, 0.05 M) containing epi-tetrahydrocorticosterone and epi-corticosterone (250 ng) (Steraloids). The homogenate was dripped slowly into pre-chilled ethanol (95%) containing glacial acetic acid (13% v/v). The sample was stored overnight at −80 °C, allowed to return to 4 °C, and sonicated chilled (1–2 min). The sample was left on ice for 20–30 min and centrifuged (20,000 × g, 20 mins, 4 °C). The supernatant was reduced to dryness under a stream of nitrogen at 60 °C, and the dried residue was dissolved in methanol (0.5 ml, 80% v/v), sonicated (1 min), and additional water (0.5 ml) was added. This solution was passed through an activated Sep-pak column (C18, Waters). The steroids were eluted from the column in methanol (2 ml) after washes with water and methanol (40% v/v). The solvent was removed under a stream of nitrogen. Conjugates were hydrolyzed, and the resulting steroids were derivatized and analyzed by GCMS as described above and previously (17). Calibration and blank standards were prepared concomitantly in the absence of tissue.

**Statistics**—All data are expressed as means ± S.E. Data were analyzed by analysis of variance followed by least squares difference (Fisher’s LSD) post hoc tests.

**RESULTS**

5α-Reduced Metabolites Bind to GR—Corticosterone and its 5α-reduced metabolites, 5αDHB and 5αTHB displaced dexamethasone in rat hepatocytes, with Kᵦ in the nM range (Fig. 2; Table 1). In contrast, 5β-reduced metabolites displaced dexamethasone...
ethasone to a minimal degree, having $K_d$ values in the $\mu M$ range.

5aTHB Activates GR in a Transient Transfection Assay—
HeLa cells were transiently co-transfected with an expression plasmid encoding human GR and LTR-luciferase (in which luciferin expression is driven by the glucocorticoid-responsive murine mammary tumor virus (MMTV) promoter). Both corticosterone and 5α-reduced corticosterone metabolites stimulated luciferase activity (Fig. 3A) was dependent upon co-transfection of glucocorticoid receptor. To test whether 5αTHB was a full or partial agonist at GR, 5αTHB was added to the incubation medium together with corticosterone. The effects upon luciferase activity were additive (Fig. 3B), suggesting that 5αTHB is a full agonist. It was not possible to measure maximal activation because high concentrations (>2 $\mu M$) of corticosterone and 5αTHB caused cell death. To eliminate the possibility that 5αTHB exerts its effects through conversion to corticosterone, the medium from cell experiments was analyzed by GCMS. Corticosterone was not detected in the medium from HeLa cells to which 5αTHB had been added and likewise 5αTHB was not detected in the medium to which corticosterone had been added (data not shown).

5αTHB Increases TAT and PEPCK mRNA in H4IIE Cells—
The experiments above relied upon GR added exogenously. To test whether 5αTHB could activate endogenous GR, the ability of 5αTHB to increase TAT and PEPCK mRNA expression was assessed in the glucocorticoid-responsive cell line, H4IIE. After 16 h of incubation with 5αTHB, TAT mRNA expression was induced, albeit to a lesser extent than by corticosterone (Fig. 4A). In the case of PEPCK, corticosterone and 5αTHB induced the transcription of mRNA of PEPCK to the same degree (Fig. 4B). Incubation of H4IIE cells with the GR antagonist RU486 had no effect alone and prevented induction of transcription of TAT and PEPCK mRNAs by 5αTHB.

5αTHB Has Glucocorticoid Effects in Vivo—To test whether 5αTHB is a GR agonist in vivo, ACTH suppression in response to a bolus of steroid was measured in adrenalecomized animals. Basal plasma ACTH levels were high, consistent with prior adrenalectomy. 1 h after steroid injection, plasma ACTH levels were suppressed in corticosterone-treated animals compared with vehicle-treated animals. Suppression of ACTH levels was also seen with 5α-THB, although the effect was smaller than that with corticosterone (Fig. 5A). Plasma corticosterone levels were increased significantly in the corticosterone-treated animals by 1 h post-injection and remained elevated at 2 h. In contrast, plasma corticosterone levels in vehicle and 5αTHB-treated animals remained low throughout the experiment (Fig. 5B).

Endogenous Concentrations of 5α-Glucocorticoid Metabolites—Similar concentrations of corticosterone (range 0.42–0.53 nmol/g tissue) and 5α-THB (range 0.14–1.2 nmol/g tissue) were measured by gas chromatography/mass spectrometry in extracts from rat liver homogenates (Fig. 6).

### Table I

| Steroid         | $K_d$ (nm) | $p = B$ |
|-----------------|-----------|---------|
| Dexamethasone   | 38 ± 8    | <0.002  |
| Corticosterone  | 153 ± 79  |         |
| 5αTHB           | 268 ± 78  | 0.33    |
| 5αDHβ           | 336 ± 142 | 0.02    |
| 5αTHB           | 448 ± 1313| <0.001  |
| 5αDHB           | 9656 ± 3230| <0.001  |

**DISCUSSION**

Our studies demonstrate that 5α-reduced glucocorticoids are present at significant concentrations in vivo and are able to compete with dexamethasone for its binding sites in hepatocytes with affinity similar to that of corticosterone. We have shown in two cell models that this binding is associated with increased transcription by GR. Further, 5αTHB has glucocorticoid activity in vivo, as judged by suppression of the hypothalamic-pituitary adrenal axis.

Binding sites for glucocorticoids other than the cytosolic GR exist in rat liver. Low affinity glucocorticoid binding sites (LAGS) have been demonstrated in the microsomal fraction (19) and nuclear envelope (20). However, the nature and function of LAGS is uncertain. Melville et al. (21) have shown that LAGS have a preference for 5α- rather than 5α-reduced steroids. It is possible that LAGS contribute to total binding in
hepatocytes presented here, so that observed $K_d$ values may be higher than we would find using purified GR.

In isolated hepatocytes, 5α-reduced metabolites of corticosterone show substantially less binding to GR than corticosterone or 5β-reduced glucocorticoids. The ability of 5α- but not 5β-reduced metabolites to bind to GR is probably due to differences in stereochemistry around the A/B ring junction. Previous studies of dexamethasone binding to cytosolic GR indicate that binding is sensitive to the configuration at the C5 position (20). The A/B ring junction of the 5α-stereoisomers is “cis” and therefore skewed relative to the plane through rings B, C, and D, whereas the 5α isozyme is “trans” and, hence, has a structure akin to corticosterone. Similar stereospecificity in favor of 5α- rather than 5β-metabolites has been observed for binding to the androgen receptor (22).

To address whether 5αTHB was not only bound to but also activated GR, we investigated GR-dependent responses in cells. We have shown in two different cell lines that 5α-reduced glucocorticoids induced GR-dependent gene transcription. First, in a transient transfection system, luciferase expression was placed under control of the MMTV promoter linked to a glucocorticoid response element (GRE). To induce translation of luciferase, ligand-activated GR must dimerize and associate with the GRE. The results indicate that the 5α-reduced metabolites were able to induce transcriptionally a glucocorticoid-sensitive reporter and that this response was dependent completely upon co-transfected GR. The effects of 5αTHB upon GR activation in this system were additive with those of corticos-

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**Fig. 4.** Induction of transcription of TAT and PEPCK mRNAs by corticosterone metabolites in H4IIE liver-derived cell line. mRNA expression of TAT (A) and PEPCK (B) measured by Northern blotting in H4IIE cells after 16-h incubation with steroid (1 μM), corticosterone, or 5αTHB in the presence or absence of the GR antagonist, RU486. Values are corrected for loading with U1. Data are mean ± S.E. of six replicates. *, $p < 0.05$ versus cells without added steroid; #, $p < 0.05$ versus steroid alone. Representative blots are shown.

**Fig. 5.** Suppression of plasma ACTH by corticosterone and 5α-tetrahydrocorticosterone in vivo. Plasma ACTH (A) and corticosterone (B) concentrations in lean male Zucker rats treated with vehicle (■), 5 mg/kg corticosterone (●), 5αTHB (▲) by intraperitoneal injection. Data are mean ± S.E. *, $p < 0.001$ versus vehicle; n = 6 per group.

**Fig. 6.** Mass chromatogram showing the presence of 5αTHB in extract from liver of a lean Zucker rat. Mass chromatogram (m/z 564) shows methoxime-trimethylsilyl derivative of epi-THB (internal standard), 5β-THB and 5αTHB, recovered from mixture of analytical standards exposed to the procedure, allowing extraction of glucocorticoid metabolites from liver (A) and liver (500 mg) of a lean Zucker rat spiked with internal standard (B).
terone, suggesting this metabolite is a full agonist. However, it was not possible to compare maximal activation of 5αTHB to that of corticosterone in this system, because cell viability was compromised at the higher concentrations of glucocorticoids that would have been necessary (>2 μM).

In a second model, H4IIE cells, activation of endogenous GR was measured by induction of transcription of endogenous TAT and PEPCK mRNAs. Expression of TAT and PEPCK mRNAs are controlled by numerous factors, including an upstream positive GRE (23, 24). Transcriptional up-regulation of both genes was observed in the presence of 5αTHB, and this was prevented by the addition of the GR antagonist, RU486.

Corticosterone was not detected after administration of 5αTHB to cell culture medium, or radioimmunoassay of plasma, corticosterone extracts of liver from lean rats, suggesting that sufficient 5αTHB was not possible to compare maximal activation of corticosterone, suggesting this metabolite is a full agonist. However, it was not possible to compare maximal activation of 5αTHB to that of corticosterone in this system, because cell viability was compromised at the higher concentrations of glucocorticoids that would have been necessary (>2 μM).

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Finally, we showed that 5αTHB administration in vivo induced a classical negative feedback effect upon the hypothalamic-pituitary-adrenal axis. In suppressing ACTH, 5αTHB had a slower onset of effect than corticosterone. This could relate in part to different central nervous system availability of 5αTHB compared with corticosterone. Although 5αTHB is bound minimally to plasma proteins (25), its access across the blood-brain barrier and its susceptibility to export from the central nervous system by multidrug resistance P-glycoproteins are unknown. Moreover, corticosterone acts through both mineralocorticoid and glucocorticoid receptors. The MR contributes more to early “shut-off” after stress and may account for the rapid effect of corticosterone (26). It is not known whether 5αTHB can bind to MR; however, 5αDHB has no mineralocorticoid activity (27).

Importantly, we found no evidence in any of our model systems of the effects of 5α-reduced corticosterone being dependent upon metabolism to corticosterone. Using GCMS analysis of cell culture medium, or radioimmunoassay of plasma, corticosterone was not detected after administration of 5αTHB. The concentrations of corticosterone and 5αTHB were similar in extracts of liver from lean rats, suggesting that sufficient 5α-reduced metabolites of corticosterone are present to influence physiological processes. The assay did not reveal the presence of 5β-reduced glucocorticoid metabolites, in keeping with data from urinary analyses reported previously, showing that the 5α-reduced glucocorticoids are the predominant A-ring-reduced metabolite in the Zucker rat (16). This result is unlike that from human urine, where equivalent 5α- and 5β-reduced metabolites of cortisol are detected.

The implication of these findings is that, when present at physiological concentrations, 5α-reduced glucocorticoids contribute to GR activation. Potentially, this will occur wherever 5α-reductases are expressed, including glucocorticoid target tissues such as liver and adipose tissue. This provides another intracrine mechanism, over and above the influence of 11β-hydroxysteroid dehydrogenases (28), whereby local concentrations of active glucocorticoids can be modulated independently of circulating corticosterone levels. These findings are of significant clinical importance because our group has demonstrated previously that 5α-reduction of glucocorticoids is increased (often at least 2-fold) in human obesity and in the Zucker rat model of obesity (16, 29). 5α-Reduction of glucocorticoids is also increased in women with polycystic ovary syndrome (30). Whether 5α-reductases contribute to GR activation and the adverse metabolic phenotype in these subjects is now a priority for further investigation.

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