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RESEARCH PAPER

5α-Reduced glucocorticoids exhibit dissociated anti-inflammatory and metabolic effects

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BACKGROUND AND PURPOSE

Dissociating anti-inflammatory efficacy from the metabolic side effects of glucocorticoids is an attractive therapeutic goal. 5α-Tetrahydro-corticosterone (5αTHB), produced from corticosterone by 5α-reductases, activates glucocorticoid receptors. This study compares the effects of 5αTHB on inflammation and metabolism *in vitro* and *in vivo*.

METHODS

Suppression of cytokine release by 5αTHB and corticosterone were studied following LPS activation of mouse bone marrow derived macrophages. *In vivo* the efficacy of these steroids to dysregulate metabolic homeostasis and modulate immune suppression and the responses to thioglycollate-induced peritonitis in C57BL/6 mice were studied following acute injection (1.5–15 mg) and chronic infusion (50 μg·day^{-1}, 14 days).

RESULTS

In macrophages, 5αTHB increased secretion of IL-10 similarly to corticosterone (180%, 340%; data are % vehicle, treated with 5αTHB and corticosterone, respectively) and suppressed LPS-induced secretion of TNF-α (21.9%, 74.2%) and IL-6 (16.4%, 69.4%). In mice with thioglycollate-induced peritonitis, both 5αTHB and corticosterone reduced the numbers of neutrophils (58.6%, 49.9%) and inflammatory monocytes (69.5%, 96.4%), and also suppressed MCP-1 (48.7%, 80.9%) and IL-6 (53.5%, 86.7%) in peritoneal exudate. In mice chronically infused with 5αTHB and corticosterone LPS-induced production of TNF-α from whole blood was suppressed to the same degree (63.2%, 37.2%). However, in contrast to corticosterone, 5αTHB did not induce body weight loss, increase blood pressure or induce hyperinsulinaemia.

CONCLUSIONS

5αTHB has anti-inflammatory effects *in vitro* and *in vivo*. At doses with equivalent anti-inflammatory efficacy to corticosterone, 5αTHB did not induce metabolic toxicity and thus may be a prototype for a safer anti-inflammatory drug.

Abbreviations

11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; ACTH, adrenocorticotrophic hormone; Agt, angiotensinogen; BMDMØ, bone marrow derived macrophage; CRH, corticotrophin releasing hormone; GR, glucocorticoid receptor; LPS, lipopolysaccharide; PEPCK, phosphoenolpyruvate carboxykinase; POMC, pro-opiomelanocortin; SGRM, selective GR modulator; TAT, tyrosine aminotransferase

Introduction

Glucocorticoids are amongst the most widely used drugs; about 1–3% of adults worldwide are taking long-term anti-inflammatory glucocorticoids (McDonough *et al.*, 2008). However, chronic systemic administration of glucocorticoids is often accompanied by a wide range of side effects, including osteoporosis, suppression of the hypothalamic-pituitary-adrenal (HPA) axis, metabolic disease (obesity, hypertension and type II diabetes) and cardiovascular disease (Walker,

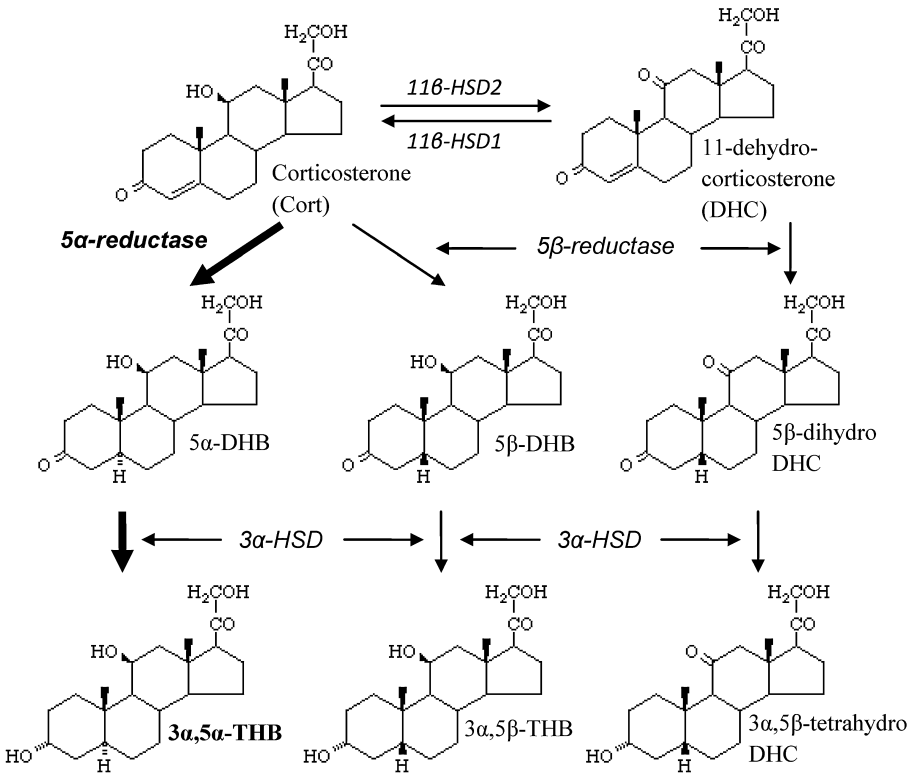

Figure 1

Metabolic pathways of corticosterone in rodents. Cort, corticosterone; DHB, dihydrocorticosterone; DHC, 11-dehydrocorticosterone; THB, tetrahydro-corticosterone; HSD, hydroxysteroid dehydrogenase.

2007; Lowenberg *et al.*, 2008). Therefore, there is substantial interest in developing new glucocorticoid receptor (GR) ligands with limited side effects. This may be possible given the diversity of mechanisms underpinning GR signalling.

The anti-inflammatory actions of glucocorticoids are in large part mediated by direct interactions between GR and other transcription factors including STAT3, promoting IL-10 expression in human B lymphocytes (Unterberger *et al.*, 2008), or NFκB and activator protein-1 (AP-1), suppressing pro-inflammatory cytokines (Smoak and Cidlowski, 2004; Serhan *et al.*, 2007). In contrast, up-regulation of many of the genes involved in metabolic toxicity, such as those encoding gluconeogenic enzymes including phosphoenolpyruvate carboxykinase (PEPCK) (Sugiyama *et al.*, 1998) and tyrosine aminotransferase (TAT) (Grange *et al.*, 1991), is mediated by GR homodimers binding to glucocorticoid response elements (GREs), inducing 'transactivation'. Suppression of the HPA axis by glucocorticoids is mediated by repression of corticotrophin releasing hormone (CRH) and pro-opiomelanocortin (POMC). Down-regulation of transcription of CRH involves direct DNA binding of monomeric GR and AP-1 at discrete adjacent sites in the promoter region (Malkoski and Dorin, 1999), whereas GRs bind as a trimer to the negative GRE in the regulatory region of POMC to inhibit gene transcription (Drouin *et al.*, 1993).

Potential selective GR modulators (SGRM) to treat inflammation, which exploit differences in mechanisms of glucocorticoid activity, have proved difficult to develop. A few 'dissociated' GR modulators have been reported (Vayssiere *et al.*, 1997; Schacke *et al.*, 2004) that selectively bind to protein transcription factors (GR-protein binding) as opposed to GR-GRE binding. These compounds show similar anti-inflammatory effects to conventional steroids but reduced side effects. However, subsequent studies revealed they were not completely dissociated *in vivo* (reviewed by Catley, 2007).

We have identified a potential SGRM from studies of endogenous steroid metabolites. Glucocorticoids in target cells are subject to metabolism by various enzymes including 11β-hydroxysteroid dehydrogenases (11β-HSDs), and the A-ring reductases (Figure 1). The principal metabolic clearance of glucocorticoids takes place in the liver by steroid A-ring reductases, including 5α- and 5β-reductases and 3α-hydroxysteroid dehydrogenases (3α-HSDs) (McInnes *et al.*, 2004). Reactions catalysed by 5α- and 5β-reductases involve the irreversible reduction of the double bond at position Δ4,5, yielding 5α- and 5β-dihydro-glucocorticoids respectively. The 5α- and 5β-reductions are followed by a further reduction by 3α-HSDs to produce 5α- and 5β-tetrahydro metabolites (Penning, 1999). This two-step A-ring reduction is common to a variety of other steroid hormones. Some 5α-reduced metabolites of steroid hormones have biological activity, including testosterone (Siiteri and Wilson, 1974), aldosterone (Kenyon *et al.*, 1983; 1985; Gorsline *et al.*, 1986) and progesterone (Smith *et al.*, 1998). Although the GR ligand binding domain preferably binds to steroids with a ketone rather than a hydroxyl at the C3 position (Bledsoe *et al.*, 2002), we previ-

ously showed that 5α-tetrahydro-corticosterone (5αTHB) can displace dexamethasone from GR binding sites in rat hepatocytes and induce transcription of a GR-regulated mouse mammary tumour virus-promoter linked reporter in transiently transfected Hela cells (McInnes *et al.*, 2004). Moreover, when 5αTHB was administered to adrenalectomized rats, it suppressed circulating adrenocorticotrophic hormone (ACTH) levels (McInnes *et al.*, 2004). Here, we aimed to explore the potential anti-inflammatory activity of 5αTHB and compare its metabolic toxicity with that of corticosterone.

Methods

Materials

Chemicals were from Sigma Aldrich, Poole, UK unless otherwise stated. Enzymes for molecular biology were from Promega, Southampton, UK. Tissue culture media were from Lonza, Verviers, Belgium. Radioactively labelled steroids were from GE Healthcare, Little Chalfont, UK. HPLC grade solvents were from Rathburn Chemicals, Walkerburn, UK and epi-steroids from Steraloids, Newport, USA.

Effects of 5αTHB in mouse bone marrow derived macrophages (BMDMØ)

Bone marrow cells were flushed from dissected femurs of male C57Bl/6 mice (Charles River, Kent, UK) and cultured for 6 days in Teflon™ pots (Roland Vetter Laborbedarf OHG, Germany) in conditioned medium consisting of L929 culture medium (20% v/v) (Hosoe *et al.*, 1989) and DMEM/F12 medium (80% v/v) supplemented with 10% fetal bovine serum, L-glutamine (200 mM), penicillin (100 U·mL^{-1}) and streptomycin (100 mg·mL^{-1}) in 5% CO_2 at 37°C. On day 6, cells were resuspended and seeded in 12-well plates at 5×10^5 cells·mL^{-1} per well and cultured overnight before use. Unstimulated BMDMØs were harvested to confirm the absence of transcripts of glucocorticoid metabolizing enzymes. In a second experiment, BMDMØs were incubated for 24 h with dexamethasone, corticosterone, 5αTHB (3 nM–1 μM) or vehicle (ethanol, 0.1% v/v) alone, or with LPS from *E coli* 011:B4 [100 ng·mL^{-1} (steroid comparison), 10 ng·mL^{-1} (dose–responses)] added 1 h after the steroid treatment. Experiments were carried out in triplicate.

Acute effects of 5αTHB in vivo

Male C57Bl/6 mice (8 weeks; body weights ≥25 g Charles River, Kent, UK) were maintained under controlled conditions of light (lights on 07:00–19:00 h) and temperature (18–20°C) and allowed access to standard chow (Special Diet Services, Witham, UK) and drinking water *ad libitum*. All animal care and experimental procedures complied with the guidelines of the UK Home Office and were approved by the University Biological Services Ethical Review Committee.

To assess steroid suppression of inflammation associated with sterile peritonitis, mice (n = 8 per group) were injected s.c. with corticosterone or 5αTHB (25, 75 or 250 mg·mL^{-1}, 20 μL) or vehicle (5% β-cyclodextrin in dimethyl sulphoxide (DMSO), 20 μL) at 09:00 h, 2 h before i.p. administration of thioglycollate (10% w/v, 300 μL) or vehicle (PBS, 300 μL). Four hours after thioglycollate injection, mice were killed, and the peritoneal cavity washed twice by injecting sterile saline (0.9%, 2 mL) and gently massaging the abdomen. Cell numbers and cell types, as well as chemokine and cytokine levels, were quantified in lavage fluid.

To assess acute induction of metabolic genes, mice (n = 3 per group) were injected s.c. with corticosterone (25, 75, 250 or 750 mg·mL^{-1}, 20 μL), 5αTHB (75 or 250 mg·mL^{-1}, 20 μL) or vehicle (DMSO, 20 μL). Mice were culled at 3 h after injection (time of peak induction by corticosterone) and livers harvested in ice-cold sucrose (250 mM)/HEPES (5 mM) buffer (pH 7.4).

Chronic effects of 5αTHB in vivo

Mini-osmotic pumps (Model 2002; Alzet®, Cupertino, USA) containing corticosterone or 5αTHB (4.165 mg·mL^{-1}, 50 μg·day^{-1}) or vehicle [DMSO and propylene glycol (1:1 v/v)] were implanted s.c. through dorsal incisions in C57BL/6 mice (n = 12 per group). Body weights and systolic blood pressure (tail cuff plethysmography, 08:00 h) were monitored and glucose tolerance tests [2 g·kg^{-1} body weight i.p. (Raubenheimer *et al.*, 2006)] performed on day 7 after mice had been deprived of food for 6 h. After 14 days, mice were decapitated, trunk blood collected immediately into tubes containing either heparin (1 U·mL^{-1}; for measurement of cytokines and steroids) or EDTA (0.5 M; for measurement of ACTH). Adipose tissue (omental, retroperitoneal, epididymal, mesenteric and subcutaneous) and organs (thymus, kidneys, adrenals, liver, spleen, brain, pituitary, right quadriceps muscle) were snap-frozen on dry ice or processed as described. Adrenals were fixed in formalin (10% v/v). Aliquots of heparin-treated blood (90 μL) were incubated with LPS (0–100 ng) in a shaking water bath (37°C, 18 h) before centrifugation (1000× g, 5 min, 4°C), and cytokines were quantified in the supernatant.

RNA extraction and PCR

Total RNA was extracted from cells and livers using TRIzol® reagent (Invitrogen, Paisley, UK), RNA from adipose tissue using RNeasy Lipid Tissue Mini Kit (Qiagen Ltd, West Sussex, UK). RNA from mouse pituitaries was extracted using an RNeasy Mini Kit (Qiagen Ltd) in combination with a QIAshredder spin column (Qiagen Ltd). cDNA was generated using a Reverse Transcription System (Promega). PCR was performed using the TaqBead™ Hot Start Polymerase kit (Promega) with initial denaturation at 95°C for 5 min and 35 cycles of PCR amplification which consisted of denaturation at 95°C for 45 s, primer annealing at 55°C for 45 s, and elongation at 72°C for 1.5 min. Upon completion, samples were incubated at 72°C for a further 5 min. The primers were: *Srd5a1* (5α-reductase 1 = NM_175283): forward 5′-CTA CAG GAG CTG CCT TCA AT-3′, reverse 5′-CTT TGC ACG TAG TGG ATC AG-3′; *Srd5a2* (5α-reductase 2 = NM_053188): forward 5′-AAC ACA GCG AGA GTG TGT CG-3′, reverse 5′-GAG AAG AGA CCC AGC AGC AC-3′; *Akr1d1* (5β-reductase = NM_145364) forward 5′-ATG GCG CCT ATG TTT ACC AC-3′, reverse 5′-ATG TGC GAC AAT GAC GAT GT-3′; *Akr1c6* (3α-HSD = NM_030611) forward 5′-AAT TGG TCC GAT CTT GCT TG-3′, reverse 5′-CCA CCC AGA TTT TGT CTC GT-3′; *Emr1* (F4/80 = NM_010130) forward 5′-AAC AAA AGT GCC CCA GTG TC-3′, reverse 5′-AGT TTG CCA TCC GGT TAC AG-3′.

Real-time PCR was performed using a LightCycler®480 (Roche Diagnostics, Mannheim, Germany) with primers (6 pmol each), corresponding 5′ FAM-labelled probe (2 pmol) and LightCycler®480 Probes Master. The assays were as follows: mouse TAT (*Tat*: NM_146214): forward 5′-GCC AGT CCG CCC ATC TG-3′, reverse 5′- TCT GGG AAG TGC TCC ATC T-3′; and TAT probe 5′-AGC CAT GTA CCT TAT GGT GGG AAT T-3′. Mouse PEPCK (*Pck1*: NM_011044): forward 5′-GTC GAA TGT GTG GGC GAT GAC-3′, reverse 5′-CTG GGT TGA TAG CCC TTA AG-3′; and PEPCK probe 5′-CCT GGA TGA AGT TTG ATG CCC AAG GC-3′. Mouse angiotensinogen (*Agt*: NM_007428): forward 5′-CTG AAC AAC ATT GGT GAC ACC-3′, reverse 5′-TGA GTT CGA GGA GGA TGC TATT-3′, and probe 5′-CCC CCG AGT GGG AGA GGT TCT-3′. The following assays were commercially designed (TaqMan® Gene Expression Assays, Applied BioSystems, UK): 11β-HSD1, Mm00476182_m1; CRH receptor 1, Mm00432670_m1; POMC, Mm00435874_m1. Negative controls omitting reverse transcriptase or RNA were included. Data were normalized for the transcript level of a housekeeping gene, cyclophilin (Mm02342429_g1), using an assay predesigned by Applied Biosystems (Warrington, UK).

Cell processing and flow cytometry analysis

Cell numbers in peritoneal lavages were quantified by haemocytometer. Peritoneal cells (300 µL) were blocked (10 min, 4°C) in mouse serum (10% v/v, Sigma Aldrich) and stained with conjugated antibodies at concentrations suggested by the supplier (30 min, 4°C). The following antibodies were used: Alexa Fluor 647 conjugated monoclonal rat-anti-mouse 7/4 (Serotec, Oxfordshire, UK), phycoerythrin (PE) conjugated rat-anti-mouse monoclonal F4/80 (Invitrogen, Paisley, UK), fluorescein isothiocyanate (FITC) conjugated monoclonal rat-anti-mouse Ly-6G and tandem Peridinin Chlorophyll Protein Complex/Cy5.5 (PerCP/Cy5.5) conjugated monoclonal rat-anti-mouse CD11b (Biolegend, CA, USA). Analyses were performed using a LSRFortessa Cell Analyser (BD Biosciences, Oxford, UK). Data analysis was performed using FlowJo software (Treestar, OR, USA).

Quantification of cytokines

Cytokines secreted into macrophage culture medium and plasma were quantified using the BD Cytometric Bead Array Mouse Inflammation Kit and measured by flow cytometry (BD FACSArray™ BioAnalyzer, BD BioSciences Immunocytometry Systems, San Jose, CA, USA). Chemokines and cytokines in peritoneal lavages were quantified using Ready-SET-Go! ELISA Kits (BD Biosciences).

Biochemical assays

Plasma glucose was quantified using an Infinity Glucose Hexokinase Liquid Stable Reagent (Thermo Electron, Melbourne, Australia), insulin using an Ultra Sensitive ELISA Kit (Crystal Chem Inc., Downers Grove, USA), triglycerides by spectrophotometry (Zen-Bio, Research Triangle Park, NC, USA); and ACTH by ELISA (Biomerica, Newport Beach, USA). Plasma corticosterone levels were measured using an in-house radioimmunoassay (Al-Dujaili *et al.*, 1981). Plasma 5αTHB was extracted, derivatized and quantified by gas chromatography/mass spectrometry (Livingstone *et al.*, 2000).

Hepatic TAT assay

Cytosol was prepared from hepatic homogenates in sucrose (250 mM)/HEPES (5 mM) buffer (pH 7.4). After centrifugation (1000× *g*, 10 min, 4°C), supernatants were further centrifuged (124 000× *g*, 45 min, 4°C), and the subsequent clear supernatant, cytosol, was retrieved. Protein concentrations were determined colorimetrically (Bio-Rad, Hemel Hempstead, UK). Cytosolic TAT activity was determined spectrophotometrically (adapted from Diamondstone, 1966). Briefly, 0.2 mL aliquots of cytosol (0.5 mg protein ml^{-1}) were pre-incubated in potassium phosphate buffer (0.2 M, pH 7.3) with tyrosine (10 M, 2.8 mL), pyridoxal phosphate (1.33 M, 0.1 mL) for 30 min at 37°C before the addition of substrate (α-ketoglutarate; 0.3 M, 0.1 mL). After a further 10 min incubation, NaOH (10 M, 0.2 mL) was added to all tubes which were then allowed to stand at room temperature for 70 min. Absorbance (λ331 nm) was determined and TAT activity ($mmol \cdot mg^{-1} \cdot min^{-1}$) calculated as 1030 × absorbance/19.9 (the absorption coefficient for the final product hydroxybenzaldehyde is 1.99 $L \cdot mmol^{-1} \cdot mm^{-1}$).

Statistics

Statistical analyses were carried out using SigmaStat 3.5 software (Dundas Software Ltd, Germany) and Minitab Statistical Software (Minitab Inc., USA). Data are mean ± SEM and were compared by one-way, two-way or repeated measures ANOVA, followed by Holm-Sidak, Dunnett's *post hoc* test, or using the General Linear Model, allowing for repeated measure with Fisher's LSD *post hoc* tests.

Results

Effects of 5αTHB in mouse bone marrow-derived macrophages in vitro

Transcripts of the A-ring reductases, that is, 3α-HSD, 5α-reductase types 1 and 2 and 5β-reductase were not detected by RT-PCR in unstimulated BMDMØs; the positive control F4/80 was detected (Figure 2A).

In the absence of LPS, 5αTHB induced IL-10 release to a similar extent as dexamethasone and corticosterone (Figure 2B); IL-6 and TNF-α release were unaffected by steroids (Figure 2C,D). When IL-10, IL-6 and TNF-α release were stimulated by LPS (Figure 2B–D), dexamethasone and corticosterone effects were inhibitory. Although weaker than dexamethasone and corticosterone, 5αTHB also inhibited LPS-induced IL-6 and TNF-α release (Figure 2C,D) but did not inhibit IL-10 release (Figure 2B).

Dose–response relationships for corticosterone and 5αTHB were explored further with respect to inhibition of LPS-induced IL-6 release (Figure 2E). After incubation with LPS (10 $ng \cdot mL^{-1}$) for 24 h, the production of IL-6 was reduced to a similar degree by corticosterone at 10 nM and by 5αTHB at 30 nM indicating a threefold difference in potency.

Acute effects of 5αTHB in vivo

In thioglycollate-induced peritonitis, 5αTHB and corticosterone reduced the total numbers of cells in peritoneal lavage fluid (Figure 3A) to a similar extent. Flow cytometric analysis

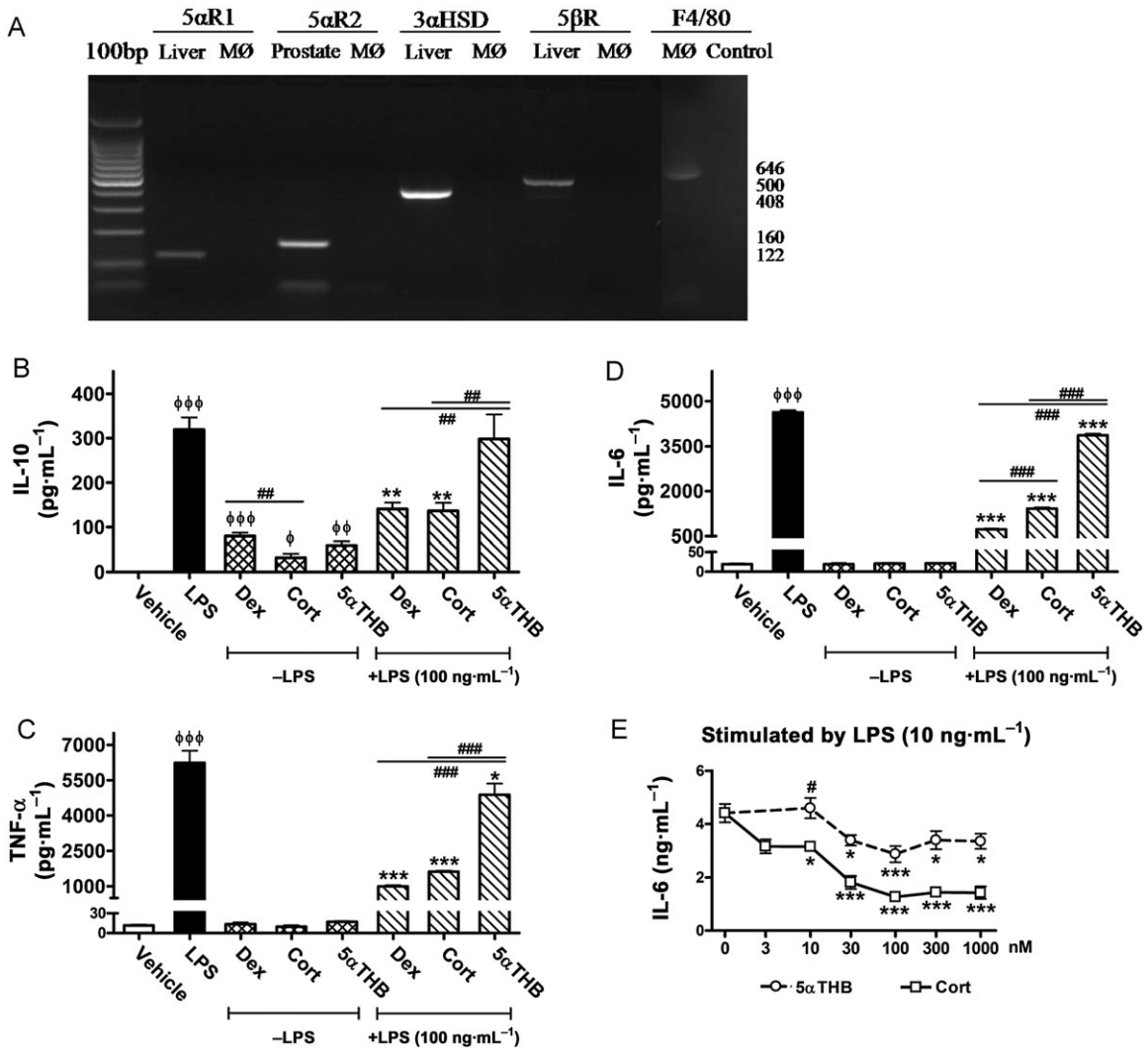

Figure 2

Anti-inflammatory effects of glucocorticoids *in vitro*. (A) Lack of expression of mRNAs of A-ring reductases in mouse bone marrow derived macrophages (BMDMØs). Total RNA was amplified by reverse transcription: 5α-reductase type 1 (5αR1) (122 bp), type 2 (5αR2) (160 bp), 3α-hydroxysteroid dehydrogenase (3αHSD) (408 bp) and 5β-reductase (5βR) (500 bp) mRNAs were not detected in unstimulated BMDMØs. Murine liver and prostate were used as positive controls for 5αR1 and 2 respectively. F4/80 (646 bp) was the positive control for intact RNA from BMDMØs. A reaction lacking reverse transcriptase was used as a negative control. (B–D) Secretion of (B) IL-10, (C) TNF-α and (D) IL-6 from BMDMØs into culture medium. Mature BMDMØs were primed by glucocorticoids [dexamethasone (Dex), corticosterone (Cort) or 5α-tetrahydro corticosterone (5αTHB), 1 μM each] for 1 h prior to stimulation with LPS (100 ng·mL^{-1}) for 24 h. Vehicle samples were BMDMØs incubated with ethanol only. Dex, Cort and 5αTHB increased the secretion of IL-10 versus vehicle. Dex and Cort, but not 5αTHB, suppressed IL-10 release following incubation with LPS. Dex and Cort markedly reduced the release of IL-6 and TNF-α, following stimulation by LPS, as did 5αTHB to a lesser extent. Data are mean ± SEM of $n = 3$ triplicates per treatment. $\phi P < 0.05$, $\phi\phi P < 0.01$, $\phi\phi\phi P < 0.001$ versus vehicle; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus LPS control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus another steroid treatment indicated by the bar; analysed by one-way ANOVA with Holm-Sidak *post hoc* tests. (E) Dose-responsive reduction in secretion of IL-6 induced by LPS (10 ng·mL^{-1}) by Cort and 5αTHB. Data are mean ± SEM, $n = 3$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus 0 nM (vehicle), $^{\#}P < 0.05$ versus 3 nM of corticosterone; analysed by Student's *t*-test.

of 7/4 and Ly6G staining revealed recruitment of distinct populations of neutrophils and inflammatory monocytes following thioglycollate injection (Figure 3B). Both 5αTHB and corticosterone reduced neutrophils by approximately 50% but these effects were not dose-related (Figure 3C). Corticosterone also reduced inflammatory monocyte infiltration in a dose-dependent manner but 5αTHB, although equipotent at low doses, did not suppress to the same maximal extent (Figure 3D). Similarly the lowest dose of 5αTHB was more potent than an equivalent dose of corticosterone in suppressing MCP-1 (Figure 3E) and IL-6 (Figure 3F) levels in peritoneal lavage fluid but higher 5αTHB doses had no additional effect, unlike the dose–response observed with corticosterone.

The dose-related acute (3 h) *in vivo* effects on hepatic TAT activity are shown in Figure 4. Corticosterone induced a maximal 3- to 3.5-fold stimulation of TAT activity with doses

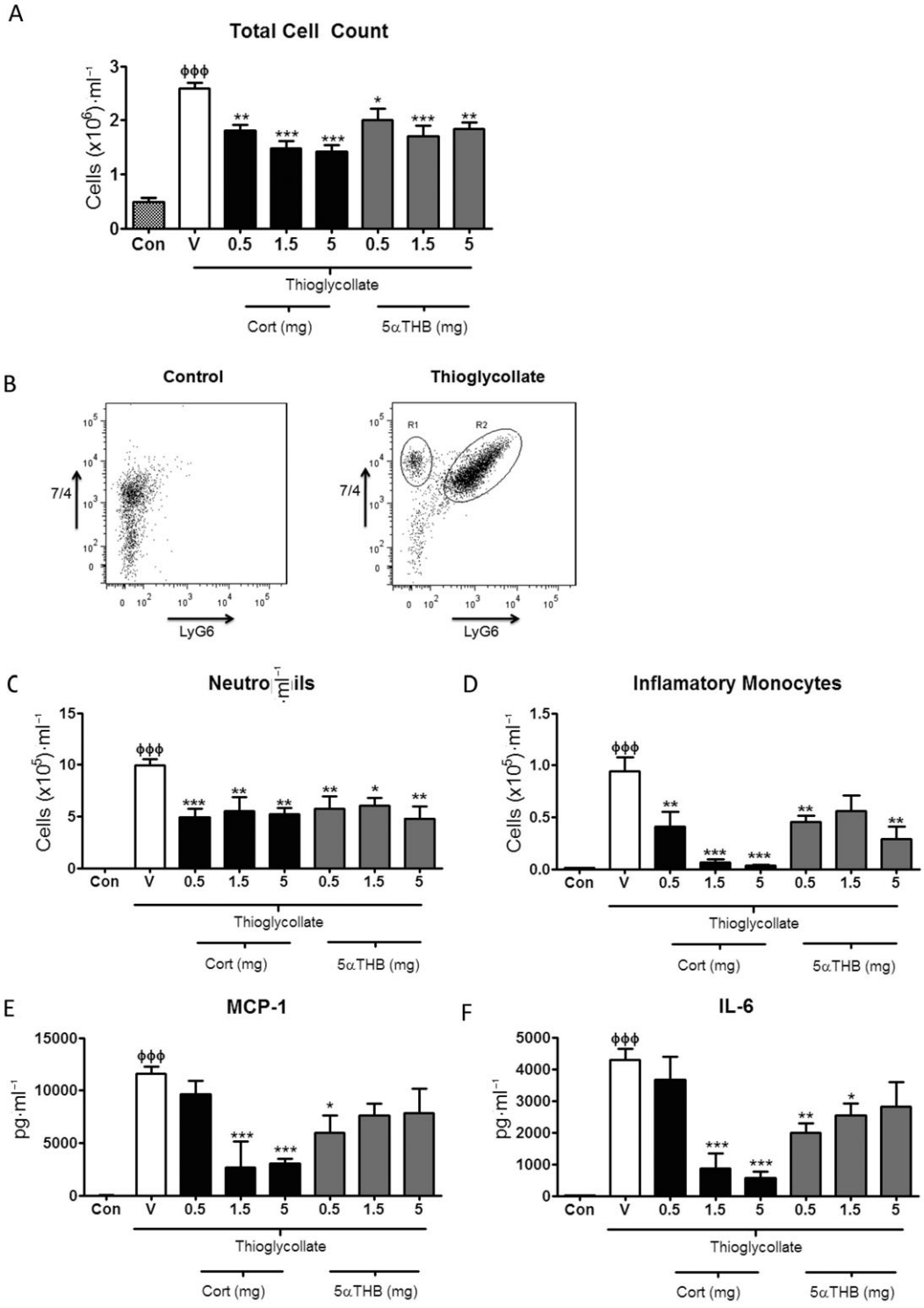

Figure 3

Acute anti-inflammatory effects of glucocorticoids *in vivo.* (A) Total cell infiltration into the peritoneum 4 h following thioglycollate-induced peritonitis was suppressed by 5αTHB and corticosterone (Cort). (B) Flow cytometric analysis of 7/4, Ly6G staining after i.p. injection of thioglycollate showing the presence of inflammatory monocytes (R1) and neutrophils (R2) versus the control profile. (C) Neutrophil infiltration into the peritoneum was suppressed to a similar extent by both Cort and 5αTHB. (D) Inflammatory monocyte infiltration was suppressed by Cort, and to a lesser extent by 5αTHB. (E) Peritoneal levels of monocyte chemoattractant protein-1 (MCP-1) were suppressed by 5αTHB, but to a lesser extent than by Cort. (F) Peritoneal levels of IL-6 were also suppressed by 5αTHB. Data are mean ± SEM, $n = 6$–8 per treatment; $\phi\phi\phi P < 0.001$ versus Control (Con); $^*P < 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ versus vehicle; analysed by one-way ANOVA with Dunnett's *post hoc* tests.

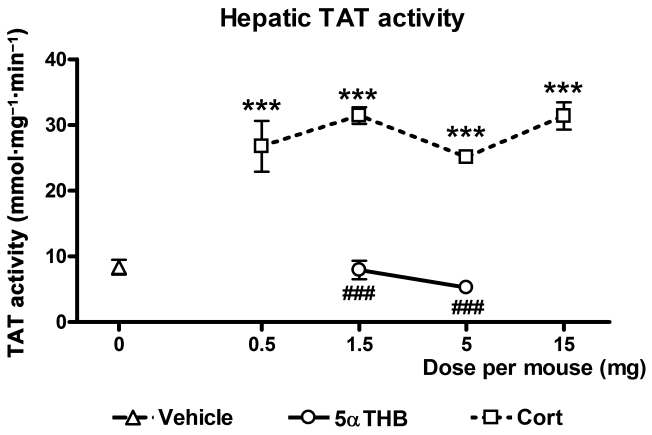

Figure 4

Acute metabolic effects of glucocorticoids *in vivo.* Corticosterone (Cort), but not 5αTHB, induced hepatic tyrosine aminotransferase (TAT) activity 3 h after injection (0.5, 1.5, 5, 15 mg, i.p.); $n = 3–5$ per treatment; $^{***}P < 0.001$ versus vehicle (no steroids treatment), $^{###}P < 0.001$ versus Cort; analysed by Student's *t*-test.

in the range 0.5–15 mg per mouse whereas 5αTHB at the dose of 1.5 and 5 mg per mouse had no effect.

Chronic effects of 5αTHB in vivo

After 2 weeks of steroid infusion, plasma corticosterone was increased only in animals receiving corticosterone infusion (Table 1). It was not possible to quantify 5αTHB in individual plasma samples because of insufficient assay sensitivity, but in samples pooled from animals in each group 5αTHB was detected only in those receiving 5αTHB infusion, at somewhat lower concentrations than corticosterone.

LPS induction of TNF-α and IL-6 in whole blood from mice treated with corticosterone and 5αTHB was suppressed to the same extent (Figure 5).

5αTHB and corticosterone had contrasting effects on body composition (Table 1). Corticosterone but not 5αTHB reduced thymus weight, decreased body weight gain and increased subcutaneous fat accumulation. Mice infused with corticosterone, but not 5αTHB, showed a significantly larger rise in blood pressure by day 11 (Table 1). Following food deprivation, basal plasma glucose was not altered by either steroid infusion, but animals infused with corticosterone showed elevated plasma insulin levels during the glucose tolerance test. 5αTHB did not affect either plasma glucose or insulin (Figure 6). Although there were no differences in hepatic glucocorticoid responsive genes, including TAT (mRNA and activity), PEPCK mRNA or angiotensinogen mRNA (Table 1), 11β-HSD1 and angiotensinogen were increased in retroperitoneal fat by corticosterone but not by 5αTHB.

Regarding the HPA axis, plasma ACTH was suppressed by both steroids, albeit somewhat less by 5αTHB than by corticosterone. However, the adrenal glands were smaller after corticosterone but not 5αTHB administration. In pituitaries, the transcript abundances of POMC and CRH receptor 1 were not significantly affected by steroid infusion.

Discussion

These data show that 5αTHB, an A-ring reduced metabolite of corticosterone, may be a prototype for an anti-inflammatory agent that lacks adverse metabolic effects. 5αTHB suppressed release of pro-inflammatory cytokines (TNF-α and IL-6) *in vitro* and *in vivo*, as well as inducing secretion of the anti-inflammatory cytokine, IL-10 *in vitro*. In an acute model of inflammation, 5αTHB, at equivalent doses to corticosterone, suppressed the infiltration of cells into the peritoneal cavity following induction of thioglycollate-induced peritonitis. Release of pro-inflammatory cytokines in LPS-stimulated whole blood was also suppressed following chronic infusion of 5αTHB. Furthermore, *in vivo* administration, either acutely or chronically, of 5αTHB did not mimic the adverse effects of corticosterone on body composition, insulin sensitivity or adrenal atrophy.

In vitro, the effects of 5αTHB on release of cytokines from BMDMØs were investigated. A-ring reductases (3α-HSD, 5α-reductase 1, 5α-reductase 2, and 5β-reductase) were not expressed in BMDMØs so these cells cannot generate 5αTHB. Moreover, there is no enzyme that converts 5αTHB into corticosterone. At equivalent doses, 5αTHB was as effective as corticosterone and dexamethasone in inducing secretion of the anti-inflammatory cytokine IL-10 from unstimulated BMDMØ and had an approximately threefold lesser effect than corticosterone in suppressing release of pro-inflammatory cytokines TNF-α and IL-6 from LPS-stimulated BMDMØ. However, 5αTHB did not suppress LPS-induced IL-10 secretion, which may reflect the relatively high dose of LPS used, or be related to subtle differences in the effect of 5αTHB on different mechanisms of GR-dependent transcription. The suppressive effects of glucocorticoids on TNF-α and IL-6 are exerted through GR-protein interactions, including NFκB and AP-1 (Smoak and Cidlowski, 2004; Serhan *et al.*, 2007), while glucocorticoid transactivates IL-10 expression through GR interaction with STAT3 (Unterberger *et al.*, 2008); both mechanisms of action were exhibited by 5αTHB. However, irrespective of the molecular mechanism, these results lend further support to the notion that 5αTHB may be an effective anti-inflammatory steroid by not directly inhibiting the generation of IL-10 (a powerful anti-inflammatory cytokine).

These effects of 5αTHB were translated *in vivo* using acute and chronic models. Following acute administration, 5αTHB suppressed the recruitment of inflammatory cells into the peritoneum in thioglycollate-induced peritonitis. Neutrophils and inflammatory monocytes are the two leucocyte populations known to be rapidly recruited to sites of infection and inflammation. 5αTHB, like corticosterone, suppressed the recruitment of neutrophils, the predominant population of infiltrating leucocytes, and suppressed peritoneal IL-6 levels, corroborating the *in vitro* findings. Interestingly, although 5αTHB suppressed recruitment of inflammatory monocytes, it had lower efficacy compared to corticosterone. Monocytes express chemokine receptor 2, which facilitates their ability to respond to the chemokine MCP-1, which was barely altered following acute administration of 5αTHB. Corticosterone treatment, in contrast, significantly reduced MCP-1 levels. This impaired ability to suppress MCP-1 release may explain the lack of

Table 1

Physiological indices and transcript abundance of genes in metabolic tissues following infusion of 5α-tetrahydro corticosterone (5αTHB) or corticosterone (50 μg·day^{-1}) or vehicle for 2 weeks

	Vehicle	5αTHB	Corticosterone
Δ Body weight (g)	0.80 ± 0.40	1.02 ± 0.32$^{\#\#}$	0.11 ± 0.34*
Indices of Hypothalamic Pituitary Adrenal axis activity			
Corticosterone (nM)	44.6 ± 13.19	59.0 ± 9.17	208.6 ± 32.00**
5αTHB (nM)	<1	13.2	<1
ACTH (pg·mL^{-1})	55.0 ± 16.9	12.9 ± 5.0*	0.8 ± 0.3**
Adrenal weight (mg)	2.0 ± 0.1	2.0 ± 0.1$^{\#\#\#}$	1.1 ± 0.1***
POMC mRNA (POMC/cyclophilin)	0.86 ± 0.15	1.2 ± 0.20	0.82 ± 0.22
CRH receptor 1 mRNA (CRH R1/cyclophilin)	0.77 ± 0.07	1.06 ± 0.25	0.64 ± 0.08
Blood pressure			
Increment in blood pressure at Day 11 (mmHg)	4.8 ± 2.2	4.7 ± 2.3$^{\#}$	14.4 ± 3.4*
Weights of immunological organs			
Thymus (mg)	30.5 ± 1.7	27.5 ± 1.6	11.7 ± 0.7**
Spleen (mg)	80.5 ± 8.6	86.9 ± 7.0	64.3 ± 7.0
Weights of metabolic organs (% of body weight)			
Liver	4.9 ± 0.2	5.0 ± 0.2	4.8 ± 0.2
Kidneys	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
Quadriceps	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Subcutaneous (s.c.) fat	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1*
Retroperitoneal (RP) fat	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Epididymal fat	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
Mesenteric fat	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Omental fat	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Hepatic TAT activity			
TAT (mmol·mg^{-1}·min^{-1})	10.9 ± 2.3	10.8 ± 1.0	11.1 ± 1.0
Transcript abundance of genes in liver (normalized for cyclophilin)			
TAT	1.70 ± 0.27	1.83 ± 0.32	2.0 ± 0.28
PEPCK	1.26 ± 0.14	1.42 ± 0.16	1.75 ± 0.23
Agt	1.44 ± 0.08	1.56 ± 0.09	1.54 ± 0.08
Transcript abundance of genes in adipose tissue (normalized for cyclophilin)			
11β-HSD1 in s.c. fat	0.75 ± 0.07	0.75 ± 0.04	1.38 ± 0.11**
11β-HSD1 in RP fat	0.84 ± 0.05	0.78 ± 0.06	1.89 ± 0.14**
Agt in s.c. fat	0.83 ± 0.19	1.01 ± 0.17	1.13 ± 0.23
Agt in RP fat	1.13 ± 0.14	1.19 ± 0.10	1.80 ± 0.27*

n = 12 per group. Data are mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 versus Cort, analysed by one-way ANOVA with Holm-Sidak *post hoc* tests.
11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; Agt, angiotensinogen; CRH, corticotrophin releasing hormone; PEPCK, phosphoenolpyruvate carboxykinase; POMC, pro-opiomelanocortin; RP, retroperitoneal; TAT, tyrosine amino transferase.

suppression of inflammatory monocytes following 5αTHB treatment.

Chronic modulation of cytokine secretion was also evident in immune suppression following infusion of 5αTHB for 2 weeks. Secretion of TNF-α and IL-6 from LPS-stimulated whole blood [probably reflecting release from monocytes (Baybutt and Holsboer, 1990)] in response to a wide range of LPS concentrations was suppressed to an equivalent degree by corticosterone and 5αTHB. This was observed despite steroid levels in the plasma being ~10-fold lower for 5αTHB than for corticosterone, reflecting more rapid *in vivo* clearance of 5αTHB, although this could only be measured in pooled samples. This observation emphasizes the potency of 5αTHB to induce immune suppression. We did not test whether

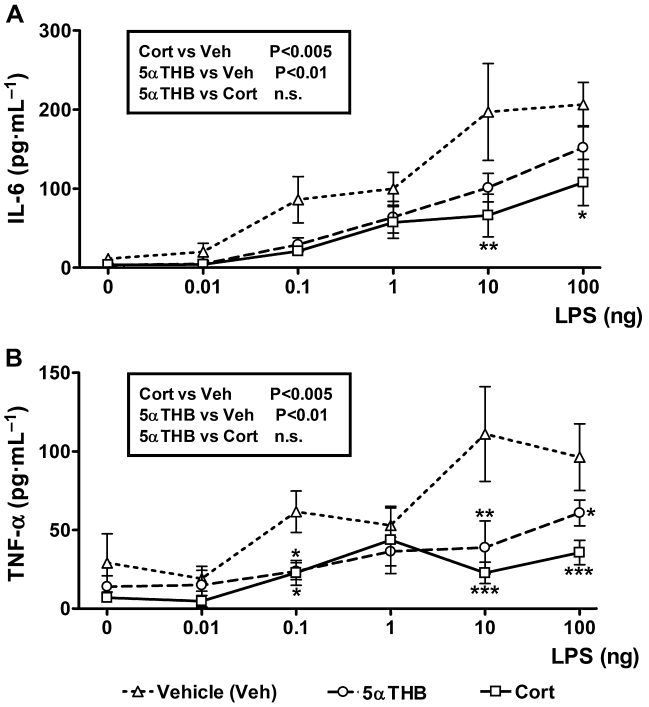

Figure 5

Chronic anti-inflammatory effects of glucocorticoids *in vivo*. (A) TNF-α and (B) IL-6 in LPS-activated whole blood collected from mice infused with corticosterone (Cort), 5α-tetrahydro corticosterone (5αTHB) or vehicle. The secretion of IL-6 and TNF-α were significantly suppressed by Cort and 5αTHB to a similar extent. Data are mean ± SEM, $n = 3–6$ per treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle, analysed by the General Linear Model, allowing for repeated measure, with Fisher's LSD *post hoc* tests.

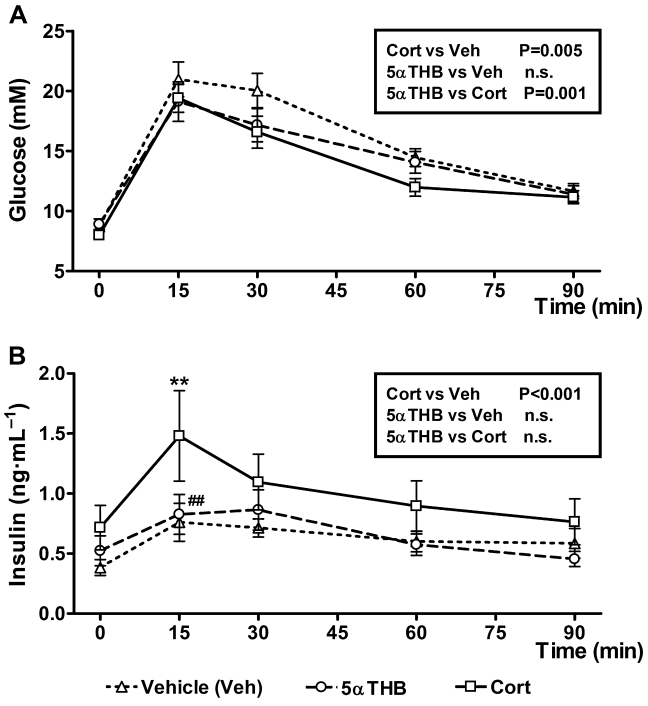

Figure 6

Chronic metabolic effects of glucocorticoids *in vivo*. (A) Plasma glucose and (B) plasma insulin during a glucose tolerance test conducted after 2 weeks' administration of corticosterone (Cort), 5α-tetrahydro corticosterone (5αTHB) or vehicle. Mice were deprived of food for 6 h before they were injected i.p. with 2 $g \cdot kg^{-1}$ body weight of glucose. Animals infused with corticosterone showed lowered plasma glucose levels and elevated plasma insulin levels versus vehicle treated animals. 5αTHB did not affect either plasma glucose or insulin. Data are mean ± SEM, $n = 12$ per group, $^{**}P < 0.01$ versus vehicle, $^{\#\#}P < 0.01$ 5αTHB versus Cort; analysed by a General Linear model, allowing for repeated measure, with Fisher's LSD *post hoc* tests.

suppression of cytokine release was maximal and thus a formal comparison of efficacy between corticosterone and 5αTHB cannot be made. The *in vivo* effects of 5αTHB could not be explained by conversion to corticosterone as plasma corticosterone concentrations with 5αTHB were the same as vehicle-treated control values.

Despite similar anti-inflammatory actions at equivalent doses, 5αTHB appeared not to have side effects on metabolism or to induce thymic involution (Pazirandeh *et al.*, 2004). Following acute administration, corticosterone, but not 5αTHB, induced hepatic activity of TAT, which catalyses a crucial step in gluconeogenesis (Grange *et al.*, 1991). Similarly, following chronic administration, the anticipated adverse metabolic responses to glucocorticoids (weight loss in rodents, increased blood pressure and insulin resistance) were observed with corticosterone, but not 5αTHB. Fat was subtly redistributed to subcutaneous depots by corticosterone and within the subcutaneous and retroperitoneal adipose depots, corticosterone up-regulated the amounts of 11β-HSD1 mRNA [a glucocorticoid responsive gene in adipose (Morton *et al.*, 2001)] and angiotensinogen (in retroperitoneal adipose tissue only). None of these effects of corticosterone was observed following infusion of 5αTHB (or vehicle). It is important to recognize that the circulating concentration of 5αTHB was 10-fold lower than that of corticosterone and perhaps different results would be obtained if their circulating concentrations, rather than infusion rates, were matched. However, corticosterone and 5αTHB were matched for anti-inflammatory efficacy in both the acute and chronic experiments, and this is the most relevant consideration when comparing toxicity.

5αTHB may not be free of side effects mediated through suppression of the HPA axis. Corticosterone suppressed ACTH levels, with associated shrinking of the adrenal glands, although measurement of transcript abundance of POMC and CRH receptor type 1 in the pituitaries proved insensitive to detect the negative feedback effects of corticosterone. 5αTHB had less striking effects, not affecting adrenal size or pituitary POMC or CRH1 mRNA; however, plasma ACTH was significantly suppressed, albeit to a lesser extent than by corticosterone. This was not mediated by any increase in corticosterone levels and is in accordance with the previous finding of McInnes *et al.* (2004), showing greater ACTH suppression by corticosterone than by 5αTHB. In addition to any central negative feedback effect, treatment with 5αTHB could have inhibited the peripheral clearance of corticosterone by competing for hepatic steroid metabolism. For example 20α-

and 20β-HSDs, and conjugating enzymes are involved in the clearance of both the hormone and its tetrahydro metabolites (Kawamura *et al.*, 1981). Competitive inhibition of corticosterone clearance may mean that lower ACTH levels are required to maintain the same levels of corticosterone.

These studies reveal the exciting possibility that 5αTHB may possess the pharmaceutically advantageous anti-inflammatory properties of endogenous glucocorticoids but none of the adverse effects on metabolism. The molecular mechanism underlying this distinction is not clear. Effects of 5αTHB to induce IL-10 as well as suppress TNF-α and IL-6 suggest signalling mediated by 'GR-protein' interaction of GR with STAT3 (Unterberger *et al.*, 2008) and also NFκB and AP-1 (Schoneveld *et al.*, 2004). The lack of effect of 5αTHB on genes encoding proteins such as TAT, PEPCK and AGT suggests that 5αTHB does not induce GR effects mediated through homodimerization and 'positive' GRE binding (Grange *et al.*, 1991; Sugiyama *et al.*, 1998). Therefore, these data justify detailed investigation of these signalling mechanisms, investigating the hypothesis that 5αTHB activates GR preferably interacting with transcription factors rather than GREs.

In conclusion, pharmacological doses of 5αTHB selectively induce glucocorticoid-like anti-inflammatory effects with limited adverse metabolic consequences when compared with equivalent doses of corticosterone. In the future, 5αTHB can be tested as a local and systemic agent for treating experimental models of rheumatoid arthritis and bronchial asthma. As a 'dissociated steroid', 5αTHB could be a safer alternative for long-term treatment of inflammatory diseases.

Acknowledgements

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Conflicts of interest

RA and BRW are inventors on a relevant patent held by the University of Edinburgh.

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