Dysregulation of glucocorticoid metabolism in murine obesity: comparable effects of leptin resistance and deficiency

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Abstract

In obese humans, metabolism of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and A-ring reduction (by 5α- and 5β-reductases) is dysregulated in a tissue specific manner. These changes have been recapitulated in leptin resistant obese Zucker rats but were not observed in high-fat fed Wistar rats. Recent data from mouse models suggest that such discrepancies may reflect differences in leptin signalling. We therefore compared glucocorticoid metabolism in murine models of leptin deficiency and resistance. Male *ob/ob* and *db/db* mice and their respective littermate controls ($n=10$–12/group) were studied at the age of 12 weeks. Enzyme activities and mRNA expression were quantified in snap-frozen tissues. The patterns of altered pathways of steroid metabolism in obesity were similar in *ob/ob* and *db/db* mice. In liver, 5β-reductase activity and mRNA were increased and 11β-HSD1 decreased in obese mice, whereas 5α-reductase 1 (5αR1) mRNA was not altered. In visceral adipose depots, 5β-reductase was not expressed, 11β-HSD1 activity was increased and 5αR1 mRNA was not altered in obesity. By contrast, in subcutaneous adipose tissue 11β-HSD1 and 5αR1 mRNA were decreased. Systematic differences were not found between *ob/ob* and *db/db* murine models of obesity, suggesting that variations in leptin signalling through the short splice variant of the Ob receptor do not contribute to dysregulation of glucocorticoid metabolism.

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Introduction

Tissue-specific dysregulation of the glucocorticoid-generating enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in rodent (Livingstone *et al.* 2000, Liu *et al.* 2003) and human obesity (Bujalska *et al.* 1997, Fraser *et al.* 1999, Rask *et al.* 2001) and the effects of 11β-HSD1 deficiency (Morton *et al.* 2004) or inhibition (Alberts *et al.* 2002, Hermanowski-Vosatka *et al.* 2005) to protect against obesity-associated metabolic dysfunction, has supported the hypothesis that variations in glucocorticoid metabolism within target tissues play an important role in the pathophysiology of Metabolic Syndrome.

In addition to 11β-HSD1, glucocorticoids are metabolised by several other enzymes (Fig. 1). In liver, glucocorticoids are inactivated by A-ring reductases (5α- and 5β-reductases and 3α-HSD) and regenerated by 11β-HSD1 (Andrew & Walker 2002). The pattern of metabolism in adipose tissue is similar, with 5α-reductase 1 (5αR1), 3α-HSD and 11β-HSD1 being expressed (Barat *et al.* 2007, Wake *et al.* 2007*b*). A-ring reductases are also dysregulated in obesity. In human obesity, whole body A-ring reduction is enhanced, as judged by urinary steroid excretion, increasing peripheral clearance of cortisol and (Andrew *et al.* 1998, Tomlinson *et al.* 2008) potentially inducing compensatory activation of the hypothalamic–pituitary–adrenal axis (Andrew *et al.* 1998, Rask *et al.* 2001, 2002). In leptin-resistant obese Zucker rats increased urinary 5α-reduced metabolites can be accounted for by up-regulation of expression and activities of hepatic A-ring reductases (Livingstone *et al.* 2005). 5αR1 is also expressed in adipose tissue but is not dysregulated in subcutaneous (s.c.) adipose in obese humans or rats (Barat *et al.* 2007, Wake *et al.* 2007*b*).

In studies of 11β-HSD1, up-regulation of enzyme expression in adipose tissue and down-regulation in liver has not been a universal finding in obesity. For example, in diet-induced obesity in mice and rats adipose 11β-HSD1 is down-regulated (Morton *et al.* 2004, Drake *et al.* 2005). Furthermore, leptin resistant and deficient mice have been shown by Liu *et al.* to have divergent changes in hepatic 11β-HSD1 activity, with expression and activity being decreased in leptin deficient *ob/ob* mice (Liu *et al.* 2003) but paradoxically increased in leptin resistant *db/db* mice (Liu *et al.* 2005). These differences may reflect the distinctions between the defects in leptin signalling in these models.

Leptin signals through several splice variants of the leptin receptor (Ob-R; Lee *et al.* 1996). The long-form of the receptor (Ob-Rb) has an intracellular domain crucial to its signalling properties via Stat3, and is predominantly expressed in the hypothalamus, where it controls appetite regulation; this

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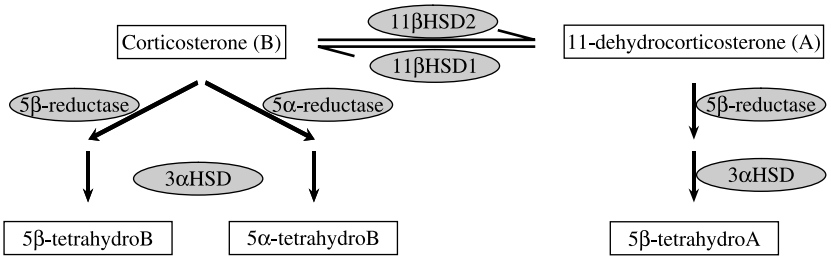

Figure 1 Glucocorticoid metabolic pathways. A is 11-dehydrocorticosterone; B is corticosterone and HSD is hydroxysteroid dehydrogenase.

cytosolic region is truncated in the *db/db* mouse (Lee *et al.* 1996, Sahai *et al.* 2004). A short form of the receptor lacking the intracellular domain (Ob-Ra) is expressed in the liver (Hoggard *et al.* 1997), where it can activate the inositol trisphosphate kinase cascade, thus potentially modulating insulin signalling pathways (Cohen *et al.* 1996, Zhao *et al.* 2000). Stimulation of Ob-Ra may occur in *db/db* mice, but not in *ob/ob* mice that lack circulating leptin. Indeed, Ob-Ra activation may be critical in the development of hepatic insulin resistance and non-alcoholic steatohepatitis (Sahai *et al.* 2004). It is possible that activation of Ob-Ra may be responsible for leptin-induced up-regulation of 11β-HSD1 expression in hepatocytes (which lack Ob-Rb; Liu *et al.* 2005), and this mechanism may mediate up-regulation of liver 11β-HSD1 in *db/db* mice. This capacity for residual leptin signalling in *db/db* mice is not predicted in Zucker obese rats, since the *fa* mutation in the leptin receptor affects the extracellular domains of both Ob-Ra and b (Chua *et al.* 1996, Da Silva *et al.* 1998). We therefore hypothesised that deficient leptin signalling underlies dysregulation of hepatic glucocorticoid metabolism by 11β-HSD1 and A-ring reductases.

The aims of the present study were to explore glucocorticoid metabolism – not only by 11β-HSD1 but also by A-ring reductases – in murine models of obesity and, by comparing findings in *ob/ob* and *db/db* mice, to dissect the potential role of Ob-Ra in mediating dysregulation of hepatic steroid metabolism in obesity.

Materials and Methods

Materials

All chemicals were obtained from Sigma unless otherwise stated. Solvents were glass distilled HPLC grade from Fisher Scientific (Loughborough, UK). Steroid standards were obtained from Steraloids (Newport, RI, USA). Radiolabelled-steroids were from GE Healthcare (Buckinghamshire, UK).

Animals

Male obese ($Lepr^{DB}/Lepr^{DB}$ (*db/db*) and Lep^{ob}/Lep^{ob} (*ob/ob*)) mice and their respective lean heterozygote or wild-type littermates (*Db/?* and *Ob/?*; C57BL background; Harlan, Bicester, UK) were characterised by phenotype, maintained under controlled conditions of light (on 0700–1900 h) and temperature (21 °C), and allowed free access to standard chow (Special Diet Services, Witham, UK) and drinking water. At 12 weeks of age, they were decapitated at 0800–1100 h and tissues dissected and snap frozen on dry ice. All animal experiments were carried out under UK Home Office guidelines.

Biochemical assays

Glucose and insulin were measured by hexokinase (Thermo Electron, Melbourne, Australia) and ELISA (Crystal Chem Inc., Downers Grove, IL, USA) respectively. Hepatic triglycerides were measured spectrophotometrically (Microgenics, Passau, Germany) as previously reported (Raubenheimer *et al.* 2006). Corticosterone was quantified in plasma by in-house RIA (Holmes *et al.* 1995).

11β-HSD1 activity assay

11β-HSD1 is a reductase *in vivo*, converting inactive 11-dehydrocorticosterone to corticosterone. However, *in vitro* dehydrogenase activity predominates and measurements of reductase activity are confounded by competition with other enzymes. Therefore, to estimate 11β-HSD1 protein, we measured enzyme activity as conversion of corticosterone to 11-dehydrocorticosterone in the presence of an excess of cofactor $NADP^+$. Aliquots of tissues were homogenised in Krebs Ringer buffer as previously described (Livingstone *et al.* 2000), and protein concentrations determined colorimetrically using a Bradford kit (Bio-Rad). Standardised amounts of protein for each tissue were incubated in duplicate at 37 °C in Krebs Ringer buffer containing 0·2% glucose, $NADP^+$ (2 mmol/l), $[^3H]_4$-corticosterone (10 nmol/l) and unlabelled corticosterone (1·99 μmol/l). Protein concentrations and incubation times were optimised for each tissue to ensure first order kinetics (liver, 25 μg/ml per h; adipose tissue, 100–200 μg/ml per h). After incubation, steroids were extracted with ethyl acetate, the organic phase evaporated under nitrogen and extracts re-solubilised in mobile phase (water:acetonitrile:methanol; 60:15:25, 1·5 ml/min). Steroids were separated by HPLC using a C18

reverse phase Symmetry column (4·6 mm, 15 cm, 5 μm; Waters, Elstree, UK) at 35 °C and quantified by on-line liquid scintillation counting.

Owing to the paucity of intra-abdominal adipose tissue in lean mice, omental adipose tissue was used to quantify enzyme activity, whereas mesenteric adipose was used to quantify transcript abundance.

5β-Reductase activity assay

Hepatic 5β-reductase (5βR) activity was assessed by the conversion of $[^3H]_4$-corticosterone to $[^3H]_4$-5β-tetrahydrocorticosterone in hepatic cytosol (Livingstone *et al.* 2005). Enzyme velocity was measured by incubating cytosol in duplicate at 37 °C, in sodium phosphate buffer (40 mmol/l Na_2PO_4, 320 mmol/l sucrose, 1 mmol/l dithiothreitol, pH 7·5) containing NADPH (1 mmol/l), glucose-6-phosphate (5 mmol/l M), glucose-6-phosphate dehydrogenase (1 unit/ml), $[^3H]_4$-corticosterone (10 nmol/l) and unlabelled corticosterone (1·99 μmol/l; Livingstone *et al.* 2005). Protein concentration and incubation period (0·5 mg/ml for 24 h) were optimised to ensure first order kinetics. Steroids were extracted with ethyl acetate, the organic phase was evaporated under nitrogen and extracts re-solubilised in mobile phase and analysed by HPLC as above.

Quantification of mRNA by real-time quantitative PCR

Total RNA was extracted from snap-frozen tissue samples, and 500 ng reverse transcribed into cDNA with random primers using the QuantiTect DNase/reverse transcription kit (Qiagen Ltd). cDNA (equivalent to 10 ng total RNA) was incubated in triplicate with 1× gene specific assay mix (Applied Biosystems, Warrington, UK) in 1× LightCycler480 Probes mastermix (Roche Diagnostics Ltd). PCR cycling and detection of fluorescent signal was carried out using a Roche LightCycler480. A standard curve was constructed for each primer probe set using a serial dilution of cDNA pooled from all samples. For liver and adipose, results were corrected for 18S and cyclophilin A RNA respectively, which were not different between groups. Assays used were: 11β-HSD1, Mm00476182_m1; 5αR1, Mm00614213_m1; 5βR, Mm00520266_m1; 18S, Hs99999 901_s1 and Cyclophilin A, Mm02342430_g1.

Statistical analysis

Data are mean ± S.E.M. and groups ($n = 10$–12 unless otherwise stated) were compared by Student's *t*-test.

Results

Both *db/db* mice and *ob/ob* mice were heavier than their respective control groups at the time of cull (Table 1), and had increased liver weight. Both *db/db* and *ob/ob* mice had higher circulating glucose, insulin and corticosterone and hepatic triglycerides than lean controls.

Hepatic glucocorticoid metabolism

Hepatic 11β-HSD1 activity was lower in both *db/db* and *ob/ob* mice compared with lean controls (Fig. 2A and B), although mRNA for 11β-HSD1 was not different in either model (Fig. 2E and F). 5βR activity and transcript abundance were higher in both *db/db* and *ob/ob* mice compared with their controls (Fig. 2C–F). There was no difference in abundance of 5αR1 mRNA between lean and obese animals of either strain (Fig. 2E and F). Activity of 5αR1 was not measured due to instability of the protein (Eicheler *et al.* 1995).

Glucocorticoid metabolism in adipose tissue

In *db/db* mice, activity of 11β-HSD1 was higher in retroperitoneal and omental adipose but lower in s.c. and epididymal adipose compared with controls (Fig. 3A). In *ob/ob* mice, 11β-HSD1 activity was higher in epididymal, retro-peritoneal and omental adipose but lower in s.c. adipose tissue compared with controls (Fig. 3B). Expression of 11β-HSD1 mRNA followed a similar pattern, in the main (Fig. 3C and D), although dysregulation of 11β-HSD1 mRNA was not observed in epididymal adipose tissue. Note that in mesenteric adipose tissue, limited amounts of tissue in lean mice resulted in analysis of only $n = 6$ samples, and hence borderline statistical significance for the up-regulation of abundance of 11β-HSD1 mRNA in *db/db* mice.

Abundance of mRNA for 5αR1 was lower in s.c. adipose tissue from obese mice of both strains compared with their respective controls, but was not altered in mesenteric,

Table 1 Body and liver weights and plasma biochemistry of mice

	***Db/?* control**	***db/db***	***Ob/?* control**	***ob/ob***
Weight at cull (g)	25·0 ± 0·54	34·5 ± 0·92*	25·7 ± 0·46	42·1 ± 0·55†
Liver (g)	1·31 ± 0·02	2·14 ± 0·14*	1·41 ± 0·08	3·79 ± 0·04†
Glucose (mg/dl)	223 ± 14	550 ± 68*	207 ± 109	367 ± 42†
Insulin (pg/ml)	1·2 ± 0·2	5·5 ± 0·9*	0·5 ± 0·1	>12·8†
Liver TAG (μmol/g)	34·7 ± 3·5	728 ± 178*	36·3 ± 5·3	2287 ± 130†
Corticosterone (nM)	7·2 ± 1·4	60·3 ± 19·5**	17·9 ± 4·7	77·4 ± 16·0†

Data are mean ± S.E.M., compared by Student's *t*-test. *$P < 0·005$, **$P < 0·01$ for *db/db* mice versus *Db/?* control. †$P < 0·005$ versus *ob/ob* mice versus *Ob/?* control. $N = 10$–13/group. NB insulin concentrations in all *ob/ob* mice exceeded the maximum point of the assay. TAG, triglycerides.

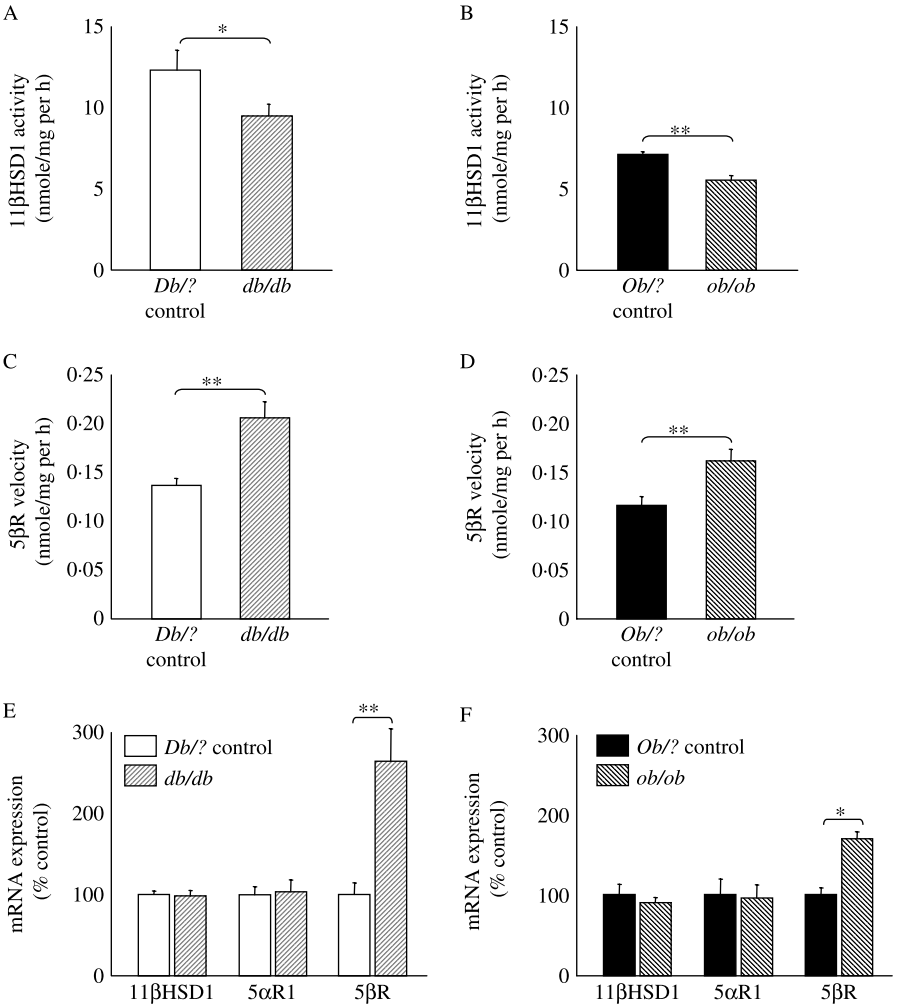

Figure 2 Hepatic glucocorticoid metabolism. 11β-HSD1 activity measured as velocity of formation of product following incubation of $[^3H]_4$-corticosterone with hepatic homogenate from (A) *Db/?* control (open) or *db/db* mice (light striped); (B) *Ob/?* control (black) or *ob/ob* mice (dark striped). 5β-Reductase activity measured as velocity of formation of product following incubation of $[^3H]_4$-corticosterone with hepatic cytosol from (C) *Db/?* control or *db/db* mice; (D) *Ob/?* control or *ob/ob* mice. Abundance of mRNAs for hepatic enzymes measured by real-time PCR (corrected for 18S as a housekeeping gene and presented as a percentage of respective control group) in (E) *Db/?* control or *db/db* mice; (F) *Ob/?* control mice or *ob/ob* mice. Data are mean±S.E.M.; $n=10$–12/group; $^*P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

epididymal or retro-peritoneal adipose tissue in either obese strain (Fig. 3E and F).

Neither 5β- nor 5α-reductase 2 mRNAs were detected in adipose tissue.

Discussion

These studies demonstrate that mice with genetic obesity due to either defective leptin secretion (*ob/ob*) or sensitivity (*db/db*) have similar alterations in 11β-HSD1 and 5βR as Zucker obese rats (Livingstone *et al.* 2005). This includes down-regulation of 11β-HSD1 in liver and up-regulation in visceral adipose tissue depots, although in obese mice 11β-HSD1 was lower in s.c. adipose depots. By contrast with Zucker rats (Livingstone *et al.* 2005), however, 5αR1 expression was not increased in liver of obese mice and was decreased in s.c. adipose tissue. Strikingly, we did not find systematic differences between glucocorticoid metabolism in leptin deficient *ob/ob* mice and leptin-resistant *db/db* mice. This contrasts with the previous reports suggesting up-regulation of 11β-HSD1 in the liver of *db/db* mice (Liu *et al.* 2005, Nakano *et al.* 2007), and suggests that enhanced signalling through the short Ob-Ra splice variant does not contribute to

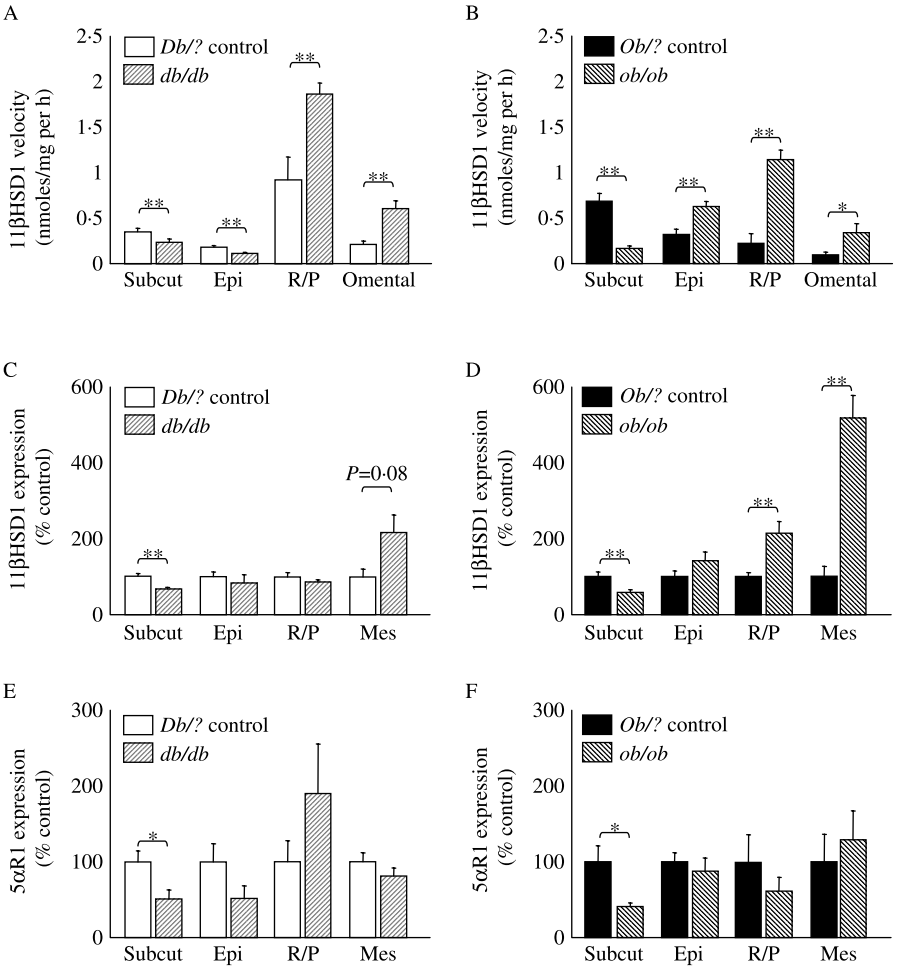

Figure 3 Glucocorticoid metabolism in adipose tissues. 11β-HSD1 activity measured as velocity of formation of product following incubation of $[^3H]_4$-corticosterone with homogenates of adipose tissues from (A) *Db/?* control (open) or *db/db* mice (light striped); (B) *Ob/?* control (black) or *ob/ob* mice (dark striped). Abundance of mRNA for 11β-HSD1 in adipose tissue depots measured by real-time PCR (corrected for cyclophilin A as a housekeeping gene and presented as a percentage of respective control group) in (C) *Db/?* control or *db/db* mice; (D) *Ob/?* control or *ob/ob* mice. Abundance of mRNAs for 5α-R1 in adipose beds measured by real-time PCR (corrected for cyclophilin A as a housekeeping gene and presented as a percentage of respective control group) in (E) *Db/?* control or *db/db* mice; (F) *Ob/?* control or *ob/ob* mice. Subcut is s.c. adipose, Epi is epididymal, R/P is retro-peritoneal and Mes is mesenteric. Data are mean ± S.E.M.; $n=6$–12/group; $^*P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

the regulation of hepatic glucocorticoid metabolism *in vivo*. Previously observed effects of leptin administration *in vivo* to reverse changes in 11β-HSD1 in *ob/ob* mice (Liu *et al*. 2003) may have been mediated indirectly through weight loss and reversal of the metabolic phenotype, which inevitably follow leptin replacement.

The pattern of dysregulation of glucocorticoid metabolism in obese rodents differs in some respects from that in obese humans. In human adipose tissue, up-regulation of s.c. 11β-HSD1 is widely reported but 5αR1 is not altered (Wake *et al*. 2007*b*) and alterations in visceral adipose 11β-HSD1 are inconsistent (Walker & Andrew 2006). In human liver, down-regulation of 11β-HSD1 and up-regulation of both 5αR1 and 5βR activity have been reported consistently (Andrew *et al*. 1998, Fraser *et al*. 1999, Rask *et al*. 2001, Tomlinson *et al*. 2008), and some of these hepatic changes are paralleled here in mice and in our studies of obese Zucker rats (Livingstone *et al*. 2000, 2005, Barat *et al*. 2007).

This species specificity provides a potential opportunity to dissect mechanisms determining dysregulation of glucocorticoid metabolism in humans using comparative studies in rodents. However, given limited knowledge of regulation of expression of A-ring reductases, the mechanism of altered A-ring reductase activity in obesity remains uncertain. 5βR is

transiently up-regulated in rats fed a high-fat diet (Drake *et al.* 2005), and in humans is selectively up-regulated in insulin resistance associated with fatty liver (Westerbacka *et al.* 2003). The murine models reported here had markedly fatty liver, moreso than that induced with diet-induced obesity. However, the severity of steatosis was more marked in the *ob/ob* mice, whereas the activity of 5β-reductase was increased to a greater extent in *db/db* mice. The *db/db* mice demonstrated partial insulin deficiency, and progression towards hyperglycaemia, whereas the *ob/ob* mice maintained near to normal glucose concentrations, albeit with higher insulin. This perhaps implicates the elevated insulin concentrations themselves in the dysregulation of steroid metabolism. The other potent regulators of 5βR identified to date are androgens, which imprint permanent down-regulation of 5βR in liver following *in utero* exposure (Einarsson & Gustafsson 1973, Gustafsson & Stenberg 1974, Jansson *et al.* 1985). In addition, withdrawal of androgens increases 5βR (Barat *et al.* 2007). Hence, the observed up-regulation may reflect the characteristic lowering of circulating androgens in obesity (Whitaker *et al.* 1983, Zumoff *et al.* 1990).

Regarding the regulation of 5αR1, these data suggest that the mechanism of dysregulation in human obesity is context and/or species-specific and does not operate in *ob/ob* or *db/db* mice. A caveat, however, is that protein levels or activity of 5αR1 might vary in the absence of changes in mRNA, but this cannot be readily tested given the instability of the hepatic 5αR1 protein *ex vivo* (Eicheler *et al.* 1995). Previous reports in both humans and rats support the notion that up-regulation of 5αR1 is secondary to the development of insulin resistance/hyperinsulinaemia and is reversible on treatment (Tsilchorozidou *et al.* 2003, Livingstone *et al.* 2005, Tomlinson *et al.* 2008). IGF-1 has been suggested as the principle candidate for dysregulation of hepatic 5αR1 (Horton *et al.* 1993). However, both mouse strains studied exhibit profound insulin resistance and therefore this explanation may be overly simplistic. Studies of 5αRs in mouse reproductive physiology have highlighted a possible redundancy between the two isozymes compared with other species (Mahendroo *et al.* 1996, 2001), but this is unlikely to explain the differences in 5αR1 dysregulation in obese mice, since the expression of 5αR2 was not detected in either liver or adipose tissue.

The few reports to date examining 5αR1 in adipose tissue in humans and Zucker rats suggest that the abundance of transcript is not altered by obesity in s.c. depots (Barat *et al.* 2007, Wake *et al.* 2007*b*). However, in both *ob/ob* and *db/db* mice, 5αR1 mRNA was down-regulated selectively in s.c. adipose, again highlighting the differences in regulation in this enzyme between species. In contrast to Zucker rats, in which 5αR1 expression was increased in omental adipose tissue (Barat *et al.* 2007), changes in mRNA expression were not observed in the murine mesenteric depot, although the greater omental depot was not studied as a direct comparison due to a paucity of tissue in lean mice.

Regulation of 11β-HSD1 transcription has been studied extensively but the basis for tissues-specific dysregulation in obesity remains elusive. Species differences in regulation of 11β-HSD1 have been demonstrated, most recently in relation to PPAR agonists (Hermanowski-Vosatka *et al.* 2000, Wake *et al.* 2007*a*). Elevated circulating glucocorticoid levels, that are much more striking in rodent than in human obesity, may contribute since 11β-HSD1 is a glucocorticoid-responsive gene (Low *et al.* 1994, Jamieson *et al.* 1995, Voice *et al.* 1996). The striking observation in murine obesity in the present data is the down-regulation of 11β-HSD1 in s.c. adipose tissue. This has also been observed in diet-induced and in polygenic obesity in mice (Morton *et al.* 2004, 2005). However in humans, inhibition of 11β-HSD1 in s.c. adipose tissue has become an attractive target for restricting glucocorticoid action, with most groups agreeing, that in humans, there is up-regulation of the enzyme in this depot (Paulmyer-Lacroix *et al.* 2002, Lindsay *et al.* 2003, Wake *et al.* 2003). Of interest is the observation that changes in 11β-HSD1 activity in both obese models are more marked than changes in mRNA, indeed in epididymal fat mRNA was not altered. This discrepancy has been reported by ourselves (Morton *et al.* 2004) and others (Bujalska *et al.* 2005, Jang *et al.* 2006) previously. The relationship between activity and abundance of transcript appears most robust in s.c. adipose in humans (Wake *et al.* 2003, Goedecke *et al.* 2006); discrepancies existing at other sites and observed here may reflect an additional level of control of 11β-HSD1 protein by post-translational modification, e.g. glycosylation (Opperman *et al.* 1995). Another source of variation between species and depots is the mixture of cell types. 11β-HSD1 is expressed in macrophages as well as adipocytes (Gilmour *et al.* 2006). There is emerging evidence that some depots, and some animal models are more susceptible to macrophage infiltration in the adipose tissue in obesity (Surmi & Hasty 2008).

In conclusion, murine obesity is characterised by some but not all of the changes in steroid metabolism that are observed in human obesity. The consequences of disrupted glucocorticoid metabolism in rodents may differ from those in humans, since rodents also exhibit substantially elevated circulating concentrations of corticosterone, contrasting with low to normal circulating cortisol in human obesity (Phillips *et al.* 2000). Nevertheless, mice may provide useful models in which to investigate dysregulation of 5βR and 11β-HSD1 but not 5αR1 in liver. None of these changes differ substantially in mice with or without leptin signalling through Ob-Ra. The pattern of dysregulation of metabolism in adipose tissue is, however, subtly different between species, offering the possibility that further comparative biology studies may elucidate relevant mechanisms.

Declaration of interest

D E W L, S L G, G L C and R A have no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported. Within the past 2 years, B R W has consulted for Astra-Zeneca, Dainippon Sumitomo, Merck, Johnson & Johnson, Incyte, Ipsen, Roche, Vitae, Wyeth, Zydus

Research Centre, received lecture fees from Abbott and Bristol Myers Squibb, and received research funding from Wyeth. B R W is an inventor on relevant patents held by University of Edinburgh.

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Author contribution statement

D E W L, S L G, G L C contributed to the execution and analysis of the studies. D E W L, B R W and R A contributed to study design, data analysis and interpretation and preparation of the manuscript.

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