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Biomarkers of hypothalamic–pituitary–adrenal axis activity in mice lacking 11β-HSD1 and H6PDH

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Abstract

Glucocorticoid concentrations are a balance between production under the negative feedback control and diurnal rhythm of the hypothalamic–pituitary–adrenal (HPA) axis and peripheral metabolism, for example by the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which catalyses the reduction of inactive cortisone (11-dehydrocorticosterone (11-DHC) in mice) to cortisol (corticosterone in mice). Reductase activity is conferred upon 11β-HSD1 by hexose-6-phosphate dehydrogenase (H6PDH). 11β-HSD1 is implicated in the development of obesity, and selective 11β-HSD1 inhibitors are currently under development. We sought to address the concern regarding potential up-regulation of the HPA axis associated with inhibition of 11β-HSD1. We assessed biomarkers for allele combinations of 11β-HSD1 and H6PDH derived from double heterozygous mouse crosses. H6PDH knock out (KO) adrenals were 69% larger than WT while 11β-HSD1 KO and double KO (DKO) adrenals were ~30% larger than WT — indicative of increased HPA axis drive in KO animals. ACTH-stimulated circulating corticosterone concentrations were 2-2-fold higher in H6PDH KO animals and ~1.5-fold higher in 11β-HSD1 KO and DKO animals compared with WT, proportional to the observed adrenal hypertrophy. KO of H6PDH resulted in a substantial increase in urinary DHC metabolites in males (65%) and females (61%). KO of 11β-HSD1 alone or in combination with H6PDH led to significant increases (36 and 42% respectively) in urinary DHC metabolites in females only. Intermediate 11β-HSD1/H6PDH heterozygotes maintained a normal HPA axis. Urinary steroid metabolite profile by gas chromatography/mass spectrometry as a biomarker assay may be beneficial in assaying HPA axis status clinically in cases of congenital and acquired 11β-HSD1/H6PDH deficiency.


Introduction

11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a 34 kDa enzyme anchored in the endoplasmic reticulum (ER) membrane, with its catalytic domain projecting into the ER lumen (Odermatt et al. 1999). In vitro, 11β-HSD1 is catalytically bidirectional, displaying both dehydrogenase – inactivation of cortisol (mice – corticosterone) to cortisone (mice – 11-dehydrocorticosterone (11-DHC)) – and o xo reductase (cortisone to cortisol) activities (Agarwal et al. 1989). Conversely, 11β-HSD2 acts as a dehydrogenase (Shams et al. 1998). In vivo, 11β-HSD1 activity has a strong, NADPH-dependent directional preference for reduction of cortisone to cortisol. This activity is facilitated by the enzyme hexose-6-phosphate dehydrogenase (H6PDH), which converts glucose-6-phosphate to 6-phosphogluconate and maintains a high NADPH/NADP⁺ ratio sufficient to permit 11β-HSD1-mediated cortisone reduction (Bujalska et al. 2005, Lavery et al. 2006, Zielinska et al. 2011). Indeed, H6PDH and 11β-HSD1 have a direct physical interaction in the ER lumen, with the N-terminal domain of H6PDH proving sufficient for the regulation of the directionality of 11β-HSD1 activity (Atanasov et al. 2008, Zhang et al. 2009). In H6PDH knock out (KO) mice, the change in NADPH provision leads to 11β-HSD1 ‘switching’ its catalytic preference in vivo from reductase to dehydrogenase activity. 11β-HSD1 and H6PDH are co-expressed in many tissues including liver, adipose and the CNS (Hewitt et al. 2005, Gomez-Sanchez et al. 2010) and function to regulate intracellular glucocorticoid (GC) availability at the pre-receptor level. There is an intimate relationship between cortisol secretion as regulated through the hypothalamic–pituitary–adrenal (HPA) axis and 11β-HSD1-mediated metabolism to preserve normal circulating concentrations (Draper & Stewart 2005). This is highlighted in congenital and acquired deficiency states. In humans, ‘apparent’ cortisone reductase deficiency (ACRD) and ‘true’ CRD is a continuum of hyperandrogenism with premature puberty...
due to mutations in H6PDH and 11β-HSD1 respectively (Lavery 2007, Lawson et al. 2011). In this regard, urinary steroidobolomics have been instrumental in differentiating ACRD from CRD. In ACRD, as in H6PDH KO mice, 11β-HSD1 assumes dehydrogenase activity, inactivating cortisol whereas in CRD there is a less severe urinary biochemistry phenotype due to a lack of 11β-HSD1 reductase activity, without a concomitant increase in dehydrogenase activity (Zielinska et al. 2011). In both, an increased cortisol clearance rate results in augmented HPA drive and a compensatory increase in adrenal cortisol secretion. A consequence of this is ACTH-mediated adrenal androgen excess driving disease presentation, which manifests in children as premature pubarche or in later life in females as hyperandrogenism and polycystic ovary syndrome (Lawson et al. 2011, Chan et al. 2011). Idkowiak et al. 2011). ACRD from CRD. In ACRD, as in H6PDH KO mice, steroidobolomics have been instrumental in differentiating ACTH-mediated adrenal androgen excess driving disease presentation, which manifests in children as premature pubarche or in later life in females as hyperandrogenism and polycystic ovary syndrome (Lavery et al. 2008a, Idkowiak et al. 2011).

11β-HSD1 and H6PDH KO mice have also been valuable in understanding the role of these enzymes in integrating GC metabolism and HPA activity. 11β-HSD1 KO mice have elevated plasma corticosterone and ACTH levels at the diurnal nadir with a prolonged corticosterone peak – indicative of abnormal HPA axis control and enhanced circadian HPA drive (Harris et al. 2001). However, this phenotype is dependent on the background strain of the mice, and when the 11β-HSD1KO is crossed in to the C57BL/6J background, the HPA defects are less pronounced, possibly as a result of increased hypothalamic GC receptor expression, increasing feedback sensitivity (Carter et al. 2009). Furthermore, as H6PDH KO mice display a switch in the directionality of 11β-HSD1 activity and have greatly enhanced GC clearance compared with the 11β-HSD1 KO mice, they display enhanced HPA axis activation, with grossly enlarged adrenal glands, and elevated nadir and zenith ACTH and corticosterone levels (Lavery et al. 2007, Rogoff et al. 2007). We have also generated 11β-HSD1/H6PDH double KO (DKO) mice to examine whether rescue of 11β-HSD1 dehydrogenase activity might prevent myopathy observed in H6PDH KO mice (Lavery et al. 2008b, Semjonous et al. 2011). DKO mice assumed an 11β-HSD1KO HPA profile while the myopathy persisted.

GCs can have detrimental effects on metabolic homeostasis, and therapeutic inhibition of 11β-HSD1 and/or H6PDH has been proposed as a potential strategy to ameliorate a range of disease states such as obesity, metabolic syndrome and hepatic steatosis (Tomlinson & Stewart 2005). 11β-HSD1 inhibitors are currently in phase II clinical trials (Boyle & Kowalski 2009), and a number of animal studies have been conducted in which 11β-HSD1 inhibitors appear to have efficacy (Alberts et al. 2003, Hermanowski-Vosatka et al. 2005). However, there is some concern that inhibition of 11β-HSD1 results in activation of the HPA axis that may negate any beneficial metabolic effects, through the increased secretion of adrenal cortisol (offsetting the deficit induced by the inhibitor) and increased adrenal androgen output. Though mice have no actual capacity to generate adrenal androgen, surrogate markers of HPA axis activity and GC metabolism can act as useful biomarkers of the potential implication of inhibitor use in humans.

We have evaluated that a urinary steroidobolomic biomarker of 11β-HSD1 activity using gas chromatography/mass spectrometry (GC/MS; Krone et al. 2010, Chan et al. 2011) assessed adrenal function using ACTH stimulations tests in all possible allele combinations of offspring derived from 11β-HSD1/H6PDH double heterozygote (HET) crosses.

Materials and Methods

Materials

ACTH was purchased from Sigma (cat. no. A-0298). The corticosterone enzyme immunoassay kit was from Enzo Life Sciences, Exeter, UK. Lithium heparin tubes were purchased from Sarstedt, Leicester, UK.

Design

Animal procedures were approved under the British Home Office Animal (Scientific Procedures) Act 1986 and conducted in accordance with our project licence and Local Ethics Committee approval. Mice were group housed under controlled temperature (21–23°C) and light (12 h light:12 h darkness cycle; lights on at 0700 h) with constant access to regular rodent chow and water. 11β-HSD1 KO and H6PDH mice and the double heterozygous crosses have previously been described in detail (Lavery et al. 2006, Semjonous et al. 2011). Briefly, 11β-HSD1 HET/H6PDH HET mice were bred to generate all conceivable intermediate genotypes. Pups were weaned at 3 weeks of age, with female pups being moved to separate holding cages. For male adrenal weight data, n=8 genotype was used and for corticosterone and ACTH stimulation testing n=12 genotype. Urine was collected from mice on a continuous basis until killing n=5–6 genotype.

Analysis of urinary steroidobolomic profile

Urinary samples were collected from male and female mice as described previously (Lavery et al. 2006). Samples were pooled from the same mouse. Using GC/MS, the ratio of 11-OH to 11-oxo metabolites was analysed, as described previously (Lavery et al. 2006, Krone et al. 2010, Semjonous et al. 2011).

Measurement of basal and ACTH-stimulated circulating corticosterone

Corticosterone was measured basally and following the administration of 1 unit (10 μg) ACTH/100 g mouse by i.p. injection. Trunk blood samples were collected in lithium heparin tubes following killing by decapitation between
and

**B**). DKO animals are essentially

A). This suggests that females have a different set point in terms of 11\(\beta\)-HSD1-mediated corticosterone metabolism.

In male and female H6PDH KO mice, we observed a significant increase in urinary 11-DHC metabolites (65% increase in H6PDH KO males compared with WT males), and this was true for all H6PDH homozygous genotypes (Fig. 1B). DKO animals are essentially a phenocopy of 11\(\beta\)-HSD1 KO for males (17-2 vs 16-8% 11-DHC metabolites) and females (48-4 vs 43% 11-DHC metabolites) as a result of the loss of dehydrogenase activity, normalising the effect of H6PDH loss on 11-DHC metabolite levels. Single or double 11\(\beta\)-HSD1/H6PDH heterozygous male and female mice have urinary 11-DHC metabolite profiles that are essentially similar to those of WT animals (\(\sim\) 10 vs 6-2% and \(\sim\) 8-3 vs 6-6% respectively; Fig. 2).

### Circulating corticosterone levels in male 11\(\beta\)-HSD1 and H6PDH KO animals

Basal 0900 h corticosterone was not different between WT, 11\(\beta\)-HSD1 KO and H6PDH KO or DKO mice. Upon ACTH stimulation, corticosterone levels were significantly increased compared with basal levels, across all genotypes (Fig. 3). 11\(\beta\)-HSD1 KO- and DKO-stimulated corticosterone levels were similar, with both achieving 1-5-fold higher stimulated levels compared with WT. By contrast, H6PDH KO-stimulated corticosterone levels were substantially (2-2-fold) higher than control and those of 11\(\beta\)-HSD1 KO (1-4-fold) or DKO (1-5-fold). However, the observed increases did not reach statistical significance.

### Adrenal weights in male 11\(\beta\)-HSD1 and H6PDH KO animals

11\(\beta\)-HSD1 KO animals had significantly (27%) larger adrenals than WT animals (Fig. 4A). H6PDH KO animals were found to have significantly (69%) larger adrenal weights than WT, 11\(\beta\)-HSD1 KO (33%) and DKO (28%) animals. DKO

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**Figure 1** (A) Urinary steroid metabolite profiles of WT vs 11\(\beta\)-HSD1 KO/DKO mice. Urine samples were collected from WT, 11\(\beta\)-HSD1 KO and DKO mice of both sexes using filter paper as in Materials and Methods section. Urinary steroids were subsequently extracted and analysed by GC/MS. Urinary steroid metabolites are shown as percentage 11-DHC metabolites as a proportion of total GC.

**Figure 2** Urinary steroid metabolite profiles of WT vs 11\(\beta\)-HSD1 HET/H6PDH HET mice. Urine samples were collected from WT and 11\(\beta\)-HSD1/H6PDH HET mice of both sexes on a daily basis using filter paper and were analysed as in (A). **\(P<0.01\) (n=4/group).
animals are essentially a phenocopy of 11β-HSD1 KO animals with significantly higher adrenal weights than WT animals (32%; Fig. 4A). Interestingly, 11β-HSD1 HET and H6PDH HET adrenal weights are similar to WT adrenal weights (2.3 and 2.4 vs 2.4 g respectively), suggesting that loss of one 11β-HSD1 or H6PDH allele is insufficient for eliciting any impact on adrenal weight (Fig. 4B). 11β-HSD1 KO/H6PDH HET adrenal weights are also significantly greater than those of WT animals (48%; Fig. 4B), as anticipated due to the complete loss of 11β-HSD1 activity, irrespective of the H6PDH genotype. 11β-HSD1 HET/H6PDH KO adrenal weights are significantly larger than those of WT (twofold) and of mice of all other genotypes.

Discussion

The loss of one or more 11β-HSD1/H6PDH alleles has a variable effect on GC metabolism, as revealed by the urinary steroidobolomic profile of mice of different 11β-HSD1/H6PDH genotypes. H6PDH KO, 11β-HSD1 KO and DKO animals were shown to have increased urinary 11-DHC levels relative to WT levels, indicative of reduced regeneration of active GCs (Semjonous et al. 2010) and our data corroborate these findings. In general, H6PDH KO animals display greater levels of urinary 11-DHC metabolites than 11β-HSD1 KO animals (Fig. 1A). 11β-HSD1 KO animals, in turn, have greater levels of urinary 11-DHC metabolites than WT animals. In the absence of H6PDH, oxoreductase activity is no longer conferred upon 11β-HSD1 and dehydrogenase activity predominates (Lavery et al. 2006), so 11-DHC metabolites rise. By contrast, 11β-HSD1 KO animals have loss of both oxoreductase and dehydrogenase activities. Basal WT urinary GC metabolites are comparable between males and females as shown in Fig. 1A. Interestingly, the effect of KO of 11β-HSD1 is sex specific, with male KO mice having the milder phenotype. Renal and colonic 11β-HSD2 activity is lower in female mice compared with male mice (Condon et al. 1997). Global 11β-HSD1 activity is also lower in female humans and rats (Low et al. 1993, Toogood et al. 2000). It is therefore conceivable that male and female mice have sex steroid-mediated differences in 11β-HSD1 and/or 11β-HSD2 activity set point that become apparent upon ablation of 11β-HSD1 activity, although the ultimate reason for the observed sexual dimorphism remains obscure.

11β-HSD1/H6PDH HETs and double HETs have a urinary 11-DHC profile that is comparable to that of WT animals (Fig. 2). Loss of one 11β-HSD1 and/or one H6PDH allele therefore seems to be insufficient for eliciting a significant change in the urinary 11-DHC profile of mice.

Adrenal weight fluctuates daily under ACTH stimulation (Harno & White 2010), and chronic ACTH drive on the adrenal gland induces hyperplasia as a mechanism to accommodate increased demand. Adrenal size is therefore a marker of the state of ACTH drive, as exemplified in individuals with congenital adrenal hyperplasia (e.g. CYP21 deficiency) where chronic ACTH drive causes hyperplasia of the adrenal cortex (Krone et al. 2005). H6PDH KO, 11β-HSD1 KO and DKO animals have enlarged adrenal glands compared with control counterparts. H6PDH KO mice have the largest adrenals, in-keeping with markedly elevated ACTH levels (Rogoff et al. 2007). By comparison, 11β-HSD1 KO and DKO have smaller adrenals and of an equivalent size to each other, reflecting the differing status of 11β-HSD1 activity (Semjonous et al. 2011). These data support previous studies that found the adrenal weights of 11β-HSD1 KO mice on a C57/Bl6J background to be significantly higher than those of WT animals (Carter et al. 2009). Although increased weight is likely to be due to

![Figure 3](image_url)  
**Figure 3** Corticosterone levels in ACTH-stimulated and -unstimulated male animals. Mice were either left unstimulated or stimulated with ACTH at 1 unit (10 μg)/100 g. 11β-HSD1 KO, H6PDH KO and DKO animals display elevated levels of circulating corticosterone relative to WT levels, which is indicative of enhanced HPA axis drive. However, the mean difference between groups were not found to be statistically significant (n>4/group).

![Figure 4](image_url)  
**Figure 4** (A) Adrenal weights of male WT vs HSD1 KO/H6PDH KO/DKO animals. Mice were killed and adrenals were dissected and weighed. **P<0.01 (n>4/group) between WT and KOs.  
**P<0.01 (n>4/group) between H6PDH KO and all other genotypes. (B) Adrenal weights of male WT vs 11β-HSD1 HET/H6PDH HET animals. Mice were killed and adrenals were dissected and weighed as in (A). **P<0.01 (n>4/group) between HSD1 KO/H6PDH HET and HSD1 HET/H6PDH KO vs all other genotypes.  
**P<0.01 (n>4/group) between HSD1 HET/H6PDH KO vs all other genotypes.
adrenal hyperplasia, we have not carried out histological analysis of adrenal tissue and cannot refute the possibility that hypertrophy is causal.

Combinations of 11β-HSD1/H6PDH heterozygous genotypes have adrenal weights that are similar to those of WT animals. Therefore, loss of one 11β-HSD1 allele and/or one H6PDH allele appears to be insufficient to elicit a significant change in adrenal weight and again concurs with the urinary data; therefore, heterozygosity has no significant impact on the HPA axis.

Circulating corticosterone levels are determined by an interplay between adrenal production, diurnal rhythm and peripheral 11β-HSD1-mediated regeneration in a range of tissues. Previous studies have shown increased circulating levels of corticosterone in 11β-HSD1 KO and H6PDH KO animals, relative to WT levels (Harris et al. 2001, Lavery et al. 2007, Rogoff et al. 2007). Other studies report no such elevation in circulating corticosterone levels, possibly as a function of the inherent variability of the measurement and the numbers assessed (Semjonous et al. 2010). For example, Harris et al. (2001) have assayed serum corticosterone levels pre- and 2h post-i.p. injection of corticosterone (5 mg/kg) – to measure HPA axis feedback. This method is not directly comparable to ours and may explain the discrepancy in the results obtained. We were unable to demonstrate differences in nadir corticosterone levels. Upon ACTH stimulation, circulating corticosterone levels were significantly elevated after 1 h in all groups. The general pattern of stimulation correlated with the degree of adrenal hyperplasia, with H6PDH KO mice having 43% higher corticosterone than 11β-HSD1 KO or DKO mice, which were also 48% elevated over WT, implying functional hyperplasia reflecting genotype. Again, these differences failed to reach statistical significance.

Overall, H6PDH KO, 11β-HSD1 KO and DKO animals appear to have increased HPA axis drive compared with WT animals. H6PDH KO animals display the most marked up-regulation of the HPA axis while 11β-HSD1 KO animals show a milder enhancement. DKO animals essentially are a phenocopy of 11β-HSD1 KO animals and HET’s have a normal HPA status. We propose a combinatorial approach to measuring HPA axis status in combination of 11β-HSD1 and/or H6PDH deficiency in vivo, including characterisation of the urinary steroidobolomic profile by GC/MS alongside measurement of circulating corticosterone levels and adrenal weights. The increased adrenal weight observed in 11β-HSD1 and H6PDH KO mice reflects the adrenal hypertrophy characteristic of clinical cases in which 11β-HSD1 is defective, as in ACRD (Cooper & Stewart 2009). There are, of course, caveats associated with extrapolating findings from murine studies to the clinical situation in humans. Indeed, there is even variation in phenotype between mouse models (Carter et al. 2009). However, it is hoped that the approach established here for profiling biomarkers of HPA axis status will prove useful in informing the optimisation of 11β-HSD1 and/or H6PDH inhibitors. It is conceivable that the therapeutic use of such inhibitors be tailored to patient requirements, on the basis of genotype and sex. A review of the animal and human data by Harno & White (2010) suggests that inhibitors will have minimal impact on the HPA axis. Concordantly, loss of one 11β-HSD1 allele plus one H6PDH allele has no discernable impact on HPA axis biomarkers; therefore, it is likely that 50% inhibition of 11β-HSD1 would have no deleterious effect on the HPA axis in either males or females. Further translational studies are required in order to clarify the effects of inhibition of 11β-HSD1 and/or H6PDH upon HPA axis status in humans.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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