Interactions between oestradiol and glucocorticoid regulatory effects on liver-specific glucocorticoid-inducible genes: possible evidence for a role of hepatic 11 β -hydroxysteroid dehydrogenase type 1

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Abstract

In vitro, 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD-1) catalyses the interconversion of active corticosterone and inert 11-dehydrocorticosterone. 11 β -HSD-1 is highly expressed in liver, where the reaction direction is 11 β -reduction, thus potentially increasing intrahepatic active glucocorticoid levels. Inhibition of 11 β -HSD-1 increases insulin sensitivity in humans *in vivo* suggesting that hepatic 11 β -HSD-1 plays a role in the maintenance or control of key glucocorticoid-regulated metabolic functions.

We have selectively repressed hepatic 11 β -HSD-1 in rats by oestradiol administration for 42 days. This nearly completely repressed hepatic 11 β -HSD-1 mRNA expression and enzyme activity and reduced expression of hepatic glucocorticoid-inducible genes including phos-

phenolpyruvate carboxykinase (PEPCK), the rate-limiting step in gluconeogenesis. Similar effects were seen after 3 weeks of oestradiol treatment. To examine whether this was due to any direct effect of oestradiol upon PEPCK, the experiment was repeated in adrenalectomised rats $\pm$ glucocorticoid replacement. In adrenalectomised rats, oestradiol did not attenuate hepatic PEPCK, whilst glucocorticoid replacement restored this action. Oestradiol did not alter hepatic metabolism of corticosterone by pathways other than 11 β -HSD-1.

These data suggest 11 β -HSD-1 plays an important role in maintaining expression of key glucocorticoid-regulated hepatic transcripts. Enzyme inhibition may provide a useful therapeutic target for manipulating glucose homeostasis.

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Introduction

11 β -Hydroxysteroid dehydrogenase (11 β -HSD) enzymes catalyse the interconversion of physiologically active glucocorticoids (cortisol, corticosterone) with their inactive 11-dehydro forms (cortisone, 11-dehydrocorticosterone) thus regulating the access of corticosteroids to intracellular receptors (Monder & White 1993, Seckl 1993). 11 β -HSD-2 is an exclusive 11 β -dehydrogenase (Rusvai & Náray-Fejes-Tóth 1993) conferring aldosterone selectivity on intrinsically non-specific mineralocorticoid receptors by rapidly inactivating glucocorticoids (Edwards *et al.* 1988, Funder *et al.* 1988). Its expression has not been demonstrated in the liver.

In contrast, 11 β -HSD-1 is a predominant 11 β -reductase, regenerating active glucocorticoids from inert 11-keto forms *in vivo*. It is widely distributed, with high expression in several organs including the liver. Although 11 β -HSD-1 shows bi-directional activity in tissue homogenates and purified microsomal fractions

in vitro (Lakshmi & Monder 1988), near exclusive 11 β -reduction has been demonstrated in both intact cells transfected with 11 β -HSD-1 cDNA (Duperrex *et al.* 1993) and primary cells in culture, including hepatocytes (Jamieson *et al.* 1995).

11 β -HSD-1 has been proposed to regulate ligand access to glucocorticoid receptors (GR) with which the enzyme is usually co-localised (Moisan *et al.* 1990, Whorwood *et al.* 1991). GR and 11 β -HSD-1 are abundant in liver. Thus although the physiological role of hepatic 11 β -HSD-1 is unclear at present, it clearly has the potential to play an important role in the regulation of glucocorticoid-dependent functions within the liver. Glucocorticoids control the transcription of many key hepatic enzymes involved in carbohydrate and fat metabolism, including phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis (Lamers *et al.* 1982, Sasaki *et al.* 1984).

11 β -HSD-1 shows pronounced sexual dimorphism in rat liver, with lower activity in females. Indeed, chronic

oestradiol (E2) administration almost completely suppresses hepatic 11 β -HSD-1 expression in both male and female rats (Lax *et al.* 1978, Low *et al.* 1993). The aim of this study was therefore to examine the contribution which reactivation of glucocorticoids by hepatic 11 β -HSD-1 makes to the expression of liver-specific glucocorticoid-modulated genes in the rat by exploiting the selective suppression of hepatic 11 β -HSD-1 by E2 *in vivo*.

Materials and Methods

Hormonal manipulations

Male Han Wistar rats (200–250 g) bred in our animal unit were maintained under controlled lighting (lights on from 0700 to 1900 h) and temperature (22 °C) with food and water freely available. Groups of rats ($n=5-10$, as in figure legends) underwent gonadectomy and either bilateral adrenalectomy (ADX) or sham-operation under halothane anaesthesia. For E2 administration, silicone elastomer capsules (1.95 mm internal diameter, 3.125 mm external diameter) (Dow Corning Corporation, Midland, MI, USA) containing 17 β -oestradiol (Sigma Chemical Co., Poole, Dorset, UK) were implanted subcutaneously. These produce levels of E2 similar to those found in adult female rat plasma (Painson *et al.* 1992). For animals treated for 42 days with E2, capsules were removed and replaced with fresh ones after 21 days. Control animals were implanted with blank capsules. ADX rats were maintained on 0.9% saline. Some ADX animals received dexamethasone (DEX) (Sigma) replacement (5 μ g/day by s.c. injection in 5% ethanol, 95% 0.9% NaCl). Controls received vehicle. Rats were killed 10, 21 or 42 days after surgery. Trunk blood was collected into heparinised tubes and plasma separated and stored at –20 °C until corticosterone RIA (MacPhee *et al.* 1989). Liver and hippocampus were removed and dissected on ice for assay of 11 β -HSD activity and an aliquot of liver was frozen on dry ice and stored at –80 °C until extraction of RNA or assay.

Quantification of 11 β -HSD activity

Tissues were homogenised in Krebs–Ringer bicarbonate buffer and assayed as described previously (Low *et al.* 1994b). Briefly, homogenates (6.25 μ g liver protein, 250 μ g hippocampal protein; chosen to lie in the initial linear part of the relationship between protein concentration and enzyme activity) were incubated with 200 μ mol/l NADP (Sigma) and 12 nmol/l [3 H]corticosterone (specific activity 84 Ci/mmol; Amersham International, Amersham, Bucks, UK) in a total volume of 250 μ l with Krebs–Ringer buffer supplemented with 0.2% BSA for 10 min at 37 °C. 11 β -Dehydrogenase activity was quantified in this assay as a measure of active enzyme since 11 β -reductase is unstable in homogenates. Steroids were

separated by HPLC with an on-line β -counter and 11 β -HSD activity expressed as conversion of corticosterone to 11-dehydrocorticosterone, after correction for apparent conversion in incubates without enzyme (less than 3%).

Quantification of corticosterone metabolism by other enzymes

To determine whether E2 effects might reflect substantial alterations in corticosterone metabolism by pathways other than 11 β -HSD, the HPLC elution profiles of steroid standards (Sigma) for the major hepatic metabolites of corticosterone were examined by ultraviolet absorbance at 254 nm. Steroids were separated on a C18 Microbondapak HPLC column (Waters, Watford, Hertfordshire, UK). The flow rate for the mobile phase (65:35 methanol: water) was 1.8 ml/min. Liver homogenates (50 μ g protein) were incubated essentially as above with no added cofactor, 200 μ mol/l NADP $^+$ or 200 μ mol/l NADPH and 4×10^{-5} mol/l unlabelled corticosterone and 12 nmol/l [3 H]corticosterone for 3 h at 37 °C. Ultraviolet absorption profiles were compared with the elution profile of the steroid standards to identify the products of corticosterone metabolism by the liver. The proportion of steroid recovered as 11-dehydrocorticosterone and corticosterone was calculated as: (c.p.m. recovered in sample/c.p.m. recovered in blank (no homogenate) incubates) $\times$ 100%.

Quantification of PEPCK activity

Ten per cent (w/v) liver homogenates were prepared at 0 °C in medium containing 250 mmol/l sucrose and 5 mmol/l Hepes, pH 7.4 and centrifuged at 3600 *g* for 10 min to sediment intact cells. Supernatant fractions were further centrifuged at 130 000 *g* for 30 min. The resulting supernatant (cytosol) was collected and stored at –70 °C until PEPCK assay. Following protein determination, PEPCK activity was assayed at 30 °C by coupling the formation of oxaloacetate with NADH oxidation in the presence of excess malate dehydrogenase (Boehringer-Mannheim UK, Lewes, East Sussex, UK), as described by Petrescu *et al.* (1979). The reaction mixture (1 ml final volume) contained 50 mmol/l Hepes (pH 6.5), 50 mmol/l NaHCO $_3$, 1 mmol/l MnCl $_2$, 0.25 mmol/l NADH, 1 mmol/l phosphoenolpyruvate, 1.5 U malate dehydrogenase and 10 μ l cytosolic liver preparation. The reaction was initiated with 0.15 mmol/l deoxy-GDP and the decrease in absorbance at 340 nm was followed for 5 min. Reaction mixture lacking bicarbonate was used as control.

Extraction and analysis of mRNA

Total RNA was extracted from liver tissue by the guanidinium thiocyanate method (Chomczynski & Sacchi 1987) and resuspended in diethylpyrocarbonate-treated water. RNA concentration and purity were assayed spectrophotometrically. Twenty microgram aliquots were

Figure 1 The effect of E2 on 11 β -HSD-1 activity and mRNA expression. (a) E2 reduces hepatic 11 β -HSD-1 activity. 11 β -HSD-1 activity (conversion of corticosterone (B) to 11-dehydrocorticosterone (A), was assayed in liver homogenates from gonadectomised male rats receiving E2 or vehicle controls for 10, 21 and 42 days. Data are expressed as a percentage ($\pm$ S.E.M.) of controls. $n=5-10$ /group. * $P<0.01$. (b) E2 repression of hepatic 11 β -HSD-1 mRNA expression in liver. Autoradiograph of Northern blots of liver RNA from gonadectomised male rats receiving E2 for 42 days and from controls receiving vehicle. $n=5$ /group. Blots were hybridised with 32 P-labelled cDNA probes to 11 β -HSD-1 mRNA and 7S RNA (loading control). No 11 β -HSD-1 mRNA was detectable in RNA from E2-treated animals. (c) Effect of E2 on hippocampal 11 β -HSD-1 activity. 11 β -HSD-1 activity was assayed in homogenates of hippocampus from gonadectomised male rats receiving E2 and from controls receiving vehicle for 21 and 42 days. Data are expressed as a percentage of activity ($\pm$ S.E.M.) in controls. $n=5-10$ /group.

separated on a 1.2% agarose gel containing 2% formaldehyde. RNA was blotted onto nitrocellulose membranes (Hybond-N, Amersham International), prehybridised in 6 ml phosphate buffer (0.2 mol/l NaH_2PO_4 , 0.6 mol/l Na_2HPO_4 , 5 mmol/l EDTA), 3 ml 20% SDS and 100 μg denatured herring testis DNA (Sigma) for 2 h at 55 $^{\circ}\text{C}$ and hybridised at 55 $^{\circ}\text{C}$ overnight in the same solution containing rat 11 β -HSD-1, PEPCK, angiotensinogen, S-adenosylmethionine synthetase (SAMS) or tyrosine aminotransferase (TAT) cDNA, labelled with [32 P]dCTP using a random primed DNA labelling kit (Boehringer-Mannheim). Three 20 min washes were carried out at room temperature in $1 \times \text{SSC}$ (0.3 mol/l NaCl, 0.03 mol/l sodium citrate), 0.1% SDS followed by a stringent wash at 55 $^{\circ}\text{C}$ for 30 min in $0.3 \times \text{SSC}$, 0.1% SDS. Filters were exposed to autoradiographic film for 1–4 days (adjusted to ensure the signal density was within the linear range). Filters were rehybridised with similarly labelled 7S cDNA or transferrin cDNA probes to control for loading, as previously described (Low *et al.* 1994a) (transferrin mRNA expression is not altered by E2 or glucocorticoid – data not shown). Optical density was determined using a computer-driven image analysis system (Seescan plc, Cambs, UK). Values are expressed as per cent of control levels.

Statistics

Data are the means $\pm$ S.E.M. of five to ten replicates (indicated in figure legends). Groups were compared by ANOVA and Newman–Keuls post-hoc test or by Student's unpaired *t*-test, as appropriate. Significance was set at $P<0.05$. Statistical tests were carried out on absolute data, but figures display data as per cent of control values to improve clarity.

Results

Effect of chronic E2 treatment on 11 β -HSD-1 activity and mRNA expression

E2 administration for 10, 21 and 42 days to gonadectomised male rats significantly decreased hepatic 11 β -HSD activity (Fig. 1a), confirming previous reports (Low *et al.* 1993). However, activity was not completely abolished as prolonged (60 min) incubation of liver homogenates from rats given E2 for 42 days showed detectable ($12.2 \pm 4.7\%$) conversion of corticosterone to 11-dehydrocorticosterone. 11 β -HSD-1 mRNA expression fell to undetectable levels after 21 and 42 days of E2 treatment (Fig. 1b). 11 β -HSD activity in the hippocampus was unaltered by E2 treatment for 21 or 42 days (Fig. 1c).

Effect of E2 on hepatic glucocorticoid-inducible gene expression

To examine the effect of attenuated hepatic 11 β -HSD-1 activity on local glucocorticoid action, expression of

Figure 2 The effect of E2 on hepatic expression of glucocorticoid-regulated genes. Data from Northern blots of four distinct transcripts in the livers of male gonadectomised rats receiving E2 or vehicle for 42 days, expressed as a percentage ($\pm$ S.E.M.) of controls. $n=5$ /group. Angio=angiotensinogen. * $P<0.05$.

mRNAs encoding the liver-specific glucocorticoid-inducible genes PEPCK, angiotensinogen, SAMS and TAT was quantified after 42 days of E2. PEPCK and angiotensinogen mRNA expression were significantly reduced by E2 administration, whilst SAMS and TAT mRNA expression were not significantly altered, although a downwards trend was noted (Fig. 2). Hepatic PEPCK activity was significantly reduced from 23.2 ± 1.8 to 17.9 ± 0.9 nmol/min per mg protein by 42 days of E2 administration.

To examine whether these effects were mediated directly or via alterations in glucocorticoid action in the liver, the effects of E2 in intact and ADX rats with and without DEX replacement were examined. Animals were killed after 21 days. ADX significantly increased hepatic 11 β -HSD-1 gene expression and activity compared with sham-operated controls (Fig. 3). E2 reduced liver 11 β -HSD-1 mRNA and activity in ADX rats, but to levels higher than in E2-treated adrenally intact rats and not significantly different from untreated controls. DEX administration to ADX rats returned 11 β -HSD-1 activity and gene expression to control levels, and E2 administration to these animals significantly decreased 11 β -HSD-1 to levels comparable to those in E2-treated adrenally intact rats.

Hepatic PEPCK mRNA levels were significantly decreased by 21 days of E2 treatment, albeit to a lesser extent than by 42 days (Fig. 4a). In ADX rats, E2 had no effect on PEPCK mRNA levels. DEX administration to ADX animals increased PEPCK mRNA levels towards control levels. E2 administration to ADX+DEX-treated animals significantly decreased PEPCK mRNA expression. PEPCK activity showed smaller changes than did its encoding mRNA, but was significantly reduced by E2 in adrenally intact rats, but not ADX rats (Fig. 4b).

Figure 3 The effect of E2, ADX and DEX on hepatic 11 β -HSD-1. (a) 11 β -HSD-1 activity in liver homogenates and (b) hepatic 11 β -HSD-1 mRNA in gonadectomised male rats receiving E2 or vehicle for 21 days following ADX $\pm$ DEX or sham-operation. Data are expressed as a percentage of sham-operated controls. $n=9-18$ /group. * $P<0.05$ compared with sham-operated controls. † $P<0.05$ compared with ADX controls. ND=not detected.

Effect of E2 on endogenous hepatic metabolism of corticosterone

To determine whether the effects of E2 upon glucocorticoid-sensitive mRNAs were due to actions upon other pathways of corticosterone metabolism in the liver, the metabolism of corticosterone in liver homogenates from E2-treated (42 days) and control animals was measured. All values were corrected for the values in blank (no homogenate) incubates. There were no differences between the steroid profiles of the control and E2-treated sample incubates, except in the quantities of corticosterone converted to 11-dehydrocorticosterone. With no added cofactor, conversion of corticosterone to 11-dehydrocorticosterone was 2.5% in controls and 0%

Figure 4 The effect of E2 on hepatic PEPCK is dependent upon adrenal steroids. (a) PEPCK mRNA and (b) PEPCK activity in livers of gonadectomized male rats receiving E2 or vehicle for 21 days following ADX $\pm$ DEX or sham-operation. Data are expressed as a percentage ($\pm$ S.E.M.) of sham-operated controls. $n=9-18$ /group. * $P<0.05$.

in E2-treated tissue. With NADP, these values were 28.6 and 0.5%, and with NADPH 6.7 and 0.1% respectively. No additional products of corticosterone metabolism were detected in any of the incubates. The recovery of [3 H]corticosterone as ([3 H]corticosterone+[3 H]11-dehydrocorticosterone) was 109 ± 13 and $107 \pm 14\%$ for control and for E2-treated sample incubates respectively.

Discussion

E2 administration reduced hepatic 11 β -HSD-1 mRNA expression to undetectable levels and markedly decreased 11 β -HSD activity, confirming previous studies (Lax *et al.*

1978, Low *et al.* 1993). Residual 11 β -HSD-1 activity is likely to be due to low levels of gene transcription not detected by Northern analysis. This effect of E2 is relatively specific to the rat liver, since hippocampal 11 β -HSD-1 was unaffected, and E2 does not reduce 11 β -HSD-2 activity in either kidney or placenta (Lax *et al.* 1978, Baggia *et al.* 1990, Low *et al.* 1993). E2 administration, as opposed to conventional liquorice-related 11 β -HSD inhibitors, was employed as a method of hepatic 11 β -HSD-1 attenuation for several reasons: (i) 11 β -HSD-2 is unaffected; (ii) the effect is relatively selective to the liver; and (iii) the inhibition achieved *in vivo* is much more marked. The mechanism of E2 down-regulation of hepatic 11 β -HSD-1 transcription is unclear, but depends partly upon sex steroid-determined patterns of growth hormone (GH) secretion and partly upon more direct effects (Low *et al.* 1994a).

Forty-two days of E2 administration clearly reduced hepatic mRNA expression of PEPCK and angiotensinogen and tended to decrease TAT and SAMS expression. The decrease in PEPCK mRNA was reflected by a fall in hepatic PEPCK activity. Throughout the study, effects were more marked upon PEPCK mRNA levels than activity. The explanation for this discrepancy is unclear, but it is not unique to this system. PEPCK is the rate-limiting enzyme in gluconeogenesis. Its over-expression in transgenic mice increases gluconeogenesis, producing hyperglycaemia and impaired glucose tolerance (Valera *et al.* 1994), whilst the recently generated 11 β -HSD-1 knockout mouse (Koteletsev *et al.* 1997) shows attenuated activation of PEPCK in response to starvation and resists hyperglycaemia with stress or obesity. Inhibition of 11 β -HSD-1 increases insulin sensitivity in humans *in vivo* (Walker *et al.* 1995) and so it was decided to study the effects on hepatic PEPCK in more detail.

Possible mechanisms for the observed effects on gene transcription include: (i) E2 may act directly to repress hepatic gene expression; (ii) E2 may alter corticosteroid metabolism in the liver. Reduced 11 β -HSD-1-mediated reactivation of 11-dehydrocorticosterone is anticipated to reduce expression of glucocorticoid-sensitive transcripts. Alternatively, 'feminisation' of the male liver may induce 5 α -reductase activity (Leblanc & Waxman 1988), thus increasing corticosterone metabolism to inactive products; and (iii) there may be other indirect effects of chronic E2 administration, as seen, for example, with effects of patterns of GH secretion on the liver (Lax *et al.* 1978, Low *et al.* 1994a).

E2 attenuation of PEPCK gene expression or activity is absent in ADX rats but is restored by glucocorticoid replacement, indicating a requirement for glucocorticoids in the E2 effects upon PEPCK. Thus direct inhibition of PEPCK by E2 or indirect effects mediated via alterations in GH secretory patterns appear unlikely. No products of corticosterone metabolism other than 11-dehydrocorticosterone were observed in liver homogenate

incubates. In particular, there was no observed increase in 5 α - or 5 β -reduction of corticosterone, suggesting that the reduced expression of specific glucocorticoid-sensitive mRNAs in liver following E2 is not due to alterations in other pathways of corticosterone metabolism.

The pattern of changes in PEPCK expression is compatible with the glucocorticoid control of PEPCK gene transcription (Lamers *et al.* 1982, Sasaki *et al.* 1984). E2 attenuation of PEPCK mRNA in intact rats was as potent as ADX alone, and in fact, PEPCK mRNA expression broadly changed in parallel with 11 β -HSD-1 mRNA and activity. This is not a general effect on hepatic gene transcription as evidenced by the lack of effect of these manipulations on transferrin mRNA levels. These data are therefore consistent with the effects of E2 being indirectly mediated via the marked loss of 11 β -HSD-1 reductase, which thus attenuates intrahepatic glucocorticoid regeneration.

In contrast with the effects observed in this study, previous reports describe glucocorticoid induction of 11 β -HSD activity in the liver *in vivo* (Low *et al.* 1994b, Walker *et al.* 1994). The discrepancy might reflect the longer duration of ADX in this study, the response of 11 β -HSD-1 gene expression to removal of glucocorticoids being an initial decrease of mRNA transcription followed by compensatory up-regulation mediated by a distinct mechanism consequent upon glucocorticoid depletion. This is perhaps more in accordance with its function, with the increase in 11 β -reductase an attempt to compensate for the lack of intrahepatic glucocorticoid. The control of 11 β -HSD-1 gene expression by glucocorticoids requires closer examination. Indeed in the tree shrew, prolonged cortisol treatment (28 days) attenuates 11 β -HSD-1 activity in the liver (Jamieson *et al.* 1997). Further complexities in the hormonal control of 11 β -HSD-1 are indicated by the apparent requirement of glucocorticoid for the near-complete repression of hepatic 11 β -HSD-1 by E2 seen in the adrenally intact animal.

This study reinforces data from 11 β -HSD-1 knockout mice (Koteletsev *et al.* 1997) and humans (Walker *et al.* 1995) indicating the potential influence of 11 β -HSD-1 on liver metabolic processes. The implications of abnormal 11 β -reductase activity for disease are unknown, but in principle an inappropriate increase in hepatic 11 β -HSD-1 activity could be a contributing factor in syndromes of central insulin resistance, including non-insulin-dependent diabetes mellitus (Fery 1994), as excess activity could increase gluconeogenesis and attenuate insulin sensitivity. The interactions between 11 β -HSD-1 and insulin activities may be mutual, since insulin decreases 11 β -HSD-1 mRNA in hepatocytes in culture (Jamieson *et al.* 1995), thus decreasing glucocorticoid regeneration and increasing insulin action. The potential to alter gluconeogenesis/insulin sensitivity by manipulating 11 β -HSD-1 activity in the liver is of clear therapeutic interest.

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