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**11β-Hydroxysteroid dehydrogenase type 1 expression in 2S FAZA hepatoma cells is hormonally regulated: a model system for the study of hepatic glucocorticoid metabolism**

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INTRODUCTION

11β-Hydroxysteroid dehydrogenase (11β-HSD) is a key enzyme in glucocorticoid metabolism, catalysing the conversion of active glucocorticoids into their inactive 11-keto metabolites, thus regulating glucocorticoid access to intracellular receptors. The type 1 isoform (11β-HSD 1) is widely distributed, with particularly high levels in liver, where accumulating evidence suggests that it acts as an 11β-reductase, regenerating active glucocorticoids. Investigation of the function and regulation of 11β-HSD 1 in liver has been hampered by the lack of hepatic cell lines which express 11β-HSD 1. Here, we describe 11β-HSD 1 mRNA expression and activity in 2S FAZA cells, a continuously cultured rat liver cell line. In intact 2S FAZA cells 11β-HSD 1 acts predominantly as a reductase, with very low dehydrogenase activity. In 2S FAZA cells 11β-HSD 1 activity and mRNA expression are regulated by hormones, with dexamethasone increasing activity and insulin, forskolin and insulin-like growth factor 1 decreasing it. Transfection of 2S FAZA cells with a luciferase reporter gene driven by the proximal promoter of the rat 11β-HSD 1 gene demonstrates that sequences which can mediate the responses to insulin, dexamethasone and forskolin all lie within 1800 bp of the transcription start site.

The liver plays a key role in the regulation of blood glucose homoeostasis via gluconeogenesis, glycogenolysis and glycogen synthesis [27]. Glucocorticoids are a major regulator of hepatic glucose metabolism [28], acting through glucocorticoid receptors, and 11β-HSD 1 has been suggested to modulate ligand access to these receptors [29–31]. Evidence is emerging that, in intact dividing cells and primary cultures of hepatocytes, 11β-HSD 1 acts predominantly as an 11β-reductase [25,32], regenerating active glucocorticoids from their 11-keto metabolites. Indeed, studies in humans have shown that inhibition of 11β-HSD increases insulin sensitivity [33], an effect thought to be mediated through the liver, presumably by attenuating intrahepatic glucocorticoid regeneration by 11β-reductase.

As yet, there are few good models for the study of the regulation of hepatic 11β-HSD 1, due to the lack of liver-derived cell lines which express 11β-HSD 1 activity or mRNA. Primary hepatocyte cultures, while useful, are limited in the extent to which they can be standardized and manipulated. We have examined a glucocorticoid- [34] and insulin-sensitive [35] rat hepatoma cell line (2S FAZA) which expresses important differentiated hepatic functions [36,37]. We have demonstrated 11β-HSD 1 expression in 2S FAZA cells and shown that it is hormonally regulated in a similar manner to 11β-HSD 1 in primary hepatocytes. Transfection experiments show that glucocorticoids, insulin and cAMP analogues act on the 11β-HSD 1 promoter within 1800 bp of the transcription start site.

**MATERIALS AND METHODS**

Materials

Cell culture reagents were obtained from Gibco BRL (Paisley, Renfrewshire, U.K.). [3H]B was obtained from Amersham International (Amersham, Bucks., U.K.). [3H]A was prepared as described [4]. Briefly, placental extract was incubated in 12 nM DTT, dithiothreitol; IGF 1, insulin-like growth factor 1. * To whom correspondence should be addressed.
[\textsuperscript{3}H]B and 1 mM NAD, [\textsuperscript{3}H]A was extracted with ethyl acetate, dried down under air and resuspended in ethanol. The purity of preparation was assessed by HPLC and was more than 95\% pure. RNA isolation kits were obtained from Qiagen Ltd, (Dorking, Surrey, U.K.). The RNase protection assay kit was obtained from Ambion Inc. (AMS Biotechnology, Witten, Oxon, U.K.). Luciferin was purchased from Promega, (Southampton). 2S FAZA cells were the kind gift of Dr. Ann Burchell, Ninewells Hospital and Medical School, Dundee, U.K.

Cells and cell culture

2S FAZA cells were maintained in McCoy’s 5A medium supplemented with 10\% (v/v) fetal-calf serum, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin and 200 \(\mu\)M \(\beta\)-glutamine. Cells were transferred into medium containing charcoal-stripped serum (prepared as described previously [25]) 24 h prior to hormone addition.

11\(\beta\)-HSD assays

Cells were plated at a density of 2 \(\times\) 10\(^4\)/60-mm-diam. plate. Upon reaching confluence hormones were added to the medium. B or A (25 nM) (consisting of 23 nM unlabelled steroid together with 2 nM [\textsuperscript{3}H]steroid) was added 24 h after the addition of hormones. Generally, 200 \(\mu\)l aliquots of medium were taken 6 h after the addition of corticosteroid, and the [\textsuperscript{3}H]steroid extracted as described previously [21]. 11\(\beta\)-HSD activity is expressed as the percentage conversion of substrate into product. Each treatment in each experiment was carried out on triplicate plates.

RNA preparation

Cells were washed with PBS and then scraped in 150 \(\mu\)l of PBS. The cells from each triplicate set of plates were pooled and the total RNA prepared using an RNeasy kit according to the manufacturer’s instructions.

RNase protection assays

A [\textsuperscript{32}P]UTP-labelled, 125-base antisense \(\beta\)-actin cRNA was made by transcription using the pTRI-\(\beta\)-Actin-125 plasmid (supplied with the RPA II kit from Ambion) and T3 RNA polymerase. A [\textsuperscript{32}P]UTP-labelled, 579 bp 11\(\beta\)-HSD 1 antisense cRNA was made by transcription using T3 RNA polymerase with pBluescript SKII containing the rat 11\(\beta\)-HSD 1 cDNA (described in [19]) linearized at a St\(\beta\)I site 579 bases from the 3‘ end.

RNase protection was carried out using the RPA II kit supplied by Ambion. RNA (10 \(\mu\)g) was mixed with 2 \(\times\) 10\(^5\) c.p.m. of 11\(\beta\)-HSD 1 cRNA and 1 \(\times\) 10\(^5\) c.p.m. of \(\beta\)-actin cRNA, precipitated, resuspended in 20 \(\mu\)l of hybridization buffer (Ambion) and hybridized by incubation at 45\°C overnight. The mixture was treated with 33000 units/ml RNase A and 10000 units/ml RNase T1 for 1 h at 37\°C. The products were precipitated and separated on a 6\% polyacrylamide gel containing 8 \(\mu\)M urea. The gel was dried and the protected cRNA detected by autoradiography. Absorbance of the autoradiographs was determined using a computer-driven image analysis system (Seescan, Cambridge). Film exposure was adjusted to ensure signal was in the linear range.

Transfections and assay of luciferase activity

The plasmids used were: pSV0L, a promoterless luciferase reporter gene [38]; pSV2L, a luciferase reporter gene driven by the simian virus 40 promoter [38]; and 11\(\beta\)/(-1800/+47)-luc, a luciferase construct containing a HindIII/PstI fragment of the rat 11\(\beta\)-HSD 1 gene (-1800 to +47 relative to the major transcription start site) which was derived from clone \(\lambda\)A described in [16] joined to the PstI/HindIII region of the pUC 18 polylinker and inserted into the HindIII site in pSV0L.

Cells were plated at 5 \(\times\) 10\(^4\) per 60-mm-diam. plate. After 24 h cells were transferred into Dulbecco’s modification of Eagle’s medium supplemented with 10\% (v/v) fetal-calf serum, 100 IU penicillin, 100 units of streptomycin and 200 \(\mu\)M \(\beta\)-glutamine. After 1 h, cells were transfected with 10 \(\mu\)g of plasmid DNA (5 \(\mu\)g of test plasmid, 5 \(\mu\)g of pGEM-3) using the calcium phosphate procedure [39]. The transfection medium/DNA precipitate was removed 24 h later and replaced with 2S FAZA medium with the specified hormones added. After a further 24 h, the cells were washed with PBS and then lysed by the addition of 150 \(\mu\)l of lysis buffer [25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol (DTT), 1 % Triton X-100, 10\% (v/v) glycerol]. The lysed cells were scraped, centrifuged briefly and the luciferase activity in the supernatant determined using a Berthold luminometer. Luciferase activity was assayed in a mixture containing 40 \(\mu\)l of cell extract, 5 \(\mu\)l of 5 mM ATP, 100 \(\mu\)l of assay buffer [20 mM tricine, 1.07 nM (MgCO\(_3\))\(_2\), 5H\(_2\)O, 2.6 mM MgSO\(_4\), 0.1 mM EDTA, 33.3 mM DTT, 0.2 mg/ml coenzyme A]. Assays were initiated by the injection of 105 \(\mu\)l of 1 mM luciferin. Light emission was measured over 10 s and relative light units (RLU)/\(\mu\)g of protein were calculated. In all experiments, treatments were carried out on triplicate plates.

Statistics

Data were analysed using ANOVA followed by the Dunnett multiple comparisons test or Fisher PLSD (probability of least squares difference) test. Significance was set at \(P<0.05\). Values are mean \pm S.E.M.

RESULTS

11\(\beta\)-HSD activity and expression in 2S FAZA cells

Assay of 11\(\beta\)-HSD in intact 2S FAZA cells showed predominantly 11\(\beta\)-reductase activity converting A added to the medium to B (21\% \pm 4\% after 4 h, 58\% \pm 4\% after 24 h). Dehydrogenase activity was detectable only after 24 h incubation (15\% \pm 4\% conversion of B into A). The presence of mRNA encoding 11\(\beta\)-HSD 1 was confirmed by RNase protection analysis (for example see Figure 1A) and by reverse-transcription PCR (results not shown).

Hormonal regulation of 11\(\beta\)-HSD in 2S FAZA cells

11\(\beta\)-HSD 1 enzyme activity and mRNA were measured in 2S FAZA cells treated for 24 h with 10 nM dexamethasone, 20 \(\mu\)g/ml insulin, 20 \(\mu\)g/ml insulin-like growth factor 1 (IGF 1), 10 \(\mu\)M forskolin or a combination of hormones, after which 11\(\beta\)-reductase activity was measured by the conversion of [\textsuperscript{3}H]A to [\textsuperscript{3}H]B in the medium over a period of 6 h (Table 1). Following 11\(\beta\)-HSD assay, cells were harvested and RNA encoding 11\(\beta\)-HSD 1 was confirmed by RNase protection analysis. Treatment with 10 nM dexamethasone led to a significant increase in 11\(\beta\)-reductase activity while 20 ng/ml IGF 1, 20 \(\mu\)g/ml insulin and 10 \(\mu\)M forskolin treatment all reduced 11\(\beta\)-reductase activity to a similar degree. The effects of adding IGF 1 together with either insulin or forskolin were similar to the individual treatments and were not additive. When dexamethasone was added in combination with either IGF 1, insulin or forskolin, then the effect of dexamethasone was dominant.

Measurement of 11\(\beta\)-HSD 1 mRNA levels by RNase protection analysis demonstrated that 11\(\beta\)-HSD 1 mRNA levels
were significantly decreased by treatment of cells with insulin, while forskolin tended to reduce mRNA levels, but this did not achieve statistical significance (Figure 1). Neither IGF 1 nor dexamethasone treatment appreciably altered the level of 11β-HSD 1 mRNA, but dexamethasone in conjunction with insulin increased the level of 11β-HSD 1 mRNA.

To determine whether insulin was acting on 11β-HSD 1 through the IGF 1 receptor, a concentration response to insulin was determined (Figure 2). The concentration of insulin for half-maximal effect was between 10^{-9} and 10^{-10} M, similar to the normal physiological concentration of insulin in plasma and the K_d of the insulin receptor [40].

**Hormonal responsiveness in 2S FAZA cells is conferred by the proximal promoter of the rat 11β-HSD 1 gene**

Transfection into 2S FAZA cells of a plasmid bearing a HindIII-PstI fragment of the rat 11β-HSD 1 proximal promoter (−1800 to +47) fused to a luciferase reporter gene demonstrated that this region contained hormone-responsive elements (Figure 3). Dexamethasone treatment of 2S FAZA cells transfected with p11β-1(−1800/+47)-luc led to significantly increased luciferase activity, whereas it had no effect on luciferase activity directed by the control plasmids pSV0L or pSV2L. Both forskolin and insulin treatment of 2S FAZA cells transfected with p11β-1(−1800/+47)-luc lowered luciferase activity in relation to cells
transfected with the control plasmids. Insulin treatment caused a slight increase in luciferase expression in cells transfected with the control plasmids, probably due to its general growth-stimulating effects at this concentration. IGF 1 had no significant effect on luciferase activity in cells transfected with any of the reporter plasmids.

**DISCUSSION**

Assay of 11β-HSD in growing 2S FAZA cells demonstrated that they expressed 11β-reductase activity with negligible dehydrogenase activity, and RNase protection analysis confirmed the presence of 11β-HSD 1 mRNA. This supports recent data showing that in intact cells transfected with 11β-HSD 1 expression vectors and in primary hepatocytes in culture, 11β-HSD 1 acts as a reductase [25,32]. In all three systems, the dehydrogenase activity of 11β-HSD 1 is very low, suggesting that, in *vivo*, the primary function of 11β-HSD 1 in hepatocytes is the regeneration of active glucocorticoids. This is in agreement with some *in vivo* data [41,42].

11β-HSD 1 activity is hormonally regulated in 2S FAZA cells and reflects the regulation seen in the liver *in vivo* and in primary cultures of hepatocytes [24,25]. In both primary hepatocytes and 2S FAZA cells short-term dexamethasone treatment increases, and insulin decreases, expression of 11β-HSD 1, with the effect of dexamethasone being dominant within physiologically meaningful concentrations. The length of hormone treatment differs between the studies, but all are short term and regulation of 11β-HSD 1 in 2S FAZA cells is similar to that in the liver and primary hepatocyte cultures. Forskolin and IGF 1 treatment both reduce 11β-HSD 1 activity in 2S FAZA cells. Forskolin reduces 11β-HSD 1 mRNA levels in 2S FAZA cells, but IGF 1 does not affect the 11β-HSD 1 mRNA levels, suggesting that it may act post-translationally. The action of forskolin suggests that hormones which act by altering intracellular cAMP levels, such as glucagon, may be involved in the regulation of 11β-HSD 1. As cell lines are much more amenable to manipulation than primary hepatocyte cultures they are the preferred system with which to investigate the molecular mechanisms involved in 11β-HSD 1 expression.

Preliminary studies in which 2S FAZA cells were transfected with 11β-1(–1800/+47)-luc show that dexamethasone, insulin and forskolin may act directly on transcription with dexamethasone increasing, and forskolin and insulin decreasing, transcription of the 11β-HSD 1 gene. The transfections also showed that sensitivity to dexamethasone, insulin and forskolin is conferred by sites within the proximal 1800 bases of 5′ flanking DNA of the 11β-HSD 1 gene. This region contains a number of protein-binding sites, several of which correspond to sites for the liver-enriched transcription factor, C/EBP, and which are protected by proteins in rat liver nuclear extracts from digestion by DNase I [43]. 2S FAZA cells, therefore, represent an ideal system for the further analysis of the 11β-HSD 1 promoter region.

In the rat, levels of A are around 50–100 nM (R. Best and J. R. Seckl, unpublished work). As A is largely unbound in the circulation and can freely cross the cell membrane, the liver has the potential to produce high intracellular levels of active B from A via 11β-HSD 1. Moreover, for most of the day, ‘free’ B levels are considerably lower than A (similarly E levels are higher than ‘free’ F in humans [42]). Thus A has considerably greater potential to influence intracellular B levels (via 11β-HSD 1) and thus glucocorticoid receptor activity, than circulating B itself for most of the day. The key pathways involved in the regulation of blood glucose by the liver are regulated by glucocorticoids together with insulin and glucagon [28]. Glucocorticoids increase the insulin activation of glucokinase [44] and are a permissive requirement for pyruvate kinase activity [28], both of which are enzymes involved in glycolysis. In the absence of other hormones, however, the overall action of glucocorticoids in the liver is to promote hepatic glucose output via gluconeogenesis, stimulating the expression of glucose-6-phosphatase, fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase [28,34], and to increase glucose storage in the liver via glycogen synthase [45]. Clearly then, the presence of hormonally regulated 11β-HSD 1 in the liver may add a further level of control of hepatic glucose output by determining the intracellular levels of active glucocorticoid. In addition, inappropriate 11β-HSD 1 amplification of glucocorticoid action may attenuate insulin sensitivity, leading to hepatic insulin resistance, hyperglycaemia and diabetes mellitus.

This cell line, expressing endogenous 11β-HSD 1, provides an excellent, easily manipulated model in which to investigate the molecular mechanisms involved in the regulation of 11β-HSD 1 and its role in determining the glucocorticoid sensitivity of hepatic function.

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