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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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) (EC 1.1.1.146) 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.

INTRODUCTION

11β-Hydroxysteroid dehydrogenase (11β-HSD) plays a key role in glucocorticoid metabolism, catalysing the oxidation of glucocorticoids [cortisol (F) in humans, corticosterone (B) in rats] to their physiologically inert 11-keto metabolites [cortisone (E) in humans, 11-dehydrocorticosterone (A) in rats] [1,2]. At least two isoforms of 11β-HSD exist [3–5]. 11β-HSD type 1 (11βHSD 1) is widely distributed with high levels of expression in liver and proximal renal tubules and lower levels elsewhere [6]. In contrast, 11β-HSD type 2 is largely restricted to aldosterone target tissues and the placenta [7,8]. 11β-HSD 2, the encoding cDNA of which has recently been cloned [7,9,10], is exclusively a dehydrogenase (glucocorticoid-inactivating) both *in vitro* and *in vivo*, and is NAD-dependent with a high affinity for its steroid substrates [4,5,7]. It has a well-characterized role in the distal nephron where it confers aldosterone selectivity on renal mineralocorticoid receptors [8,11], and in the placenta where it acts as a barrier to maternal glucocorticoids [12,13].

11β-HSD 1, a lower-affinity NADP(H)-dependent enzyme, has been purified from rat liver [14] and the corresponding cDNA and gene have been cloned [15,16]. Homologues have been isolated from a variety of species [6,17–19]. The highest levels of 11β-HSD 1 are found in the liver [6] where it is hormonally regulated in a sex- [20,21] and development-dependent [17,22] manner. Oestradiol treatment reduces 11β-HSD 1 mRNA expression in the liver [20,23], partly explaining the sexual dimorphism of 11β-HSD 1 expression in the rat (higher levels in male liver). This effect is thought to be mediated by the sex-specific secretion of growth hormone [21]. There is evidence that 11β-HSD 1 expression in liver is up-regulated by glucocorticoids [24,25], suggesting that glucocorticoids may modulate their own metabolism, while insulin has been shown to reduce 11β-HSD 1 expression in hepatocytes and fibroblasts [25,26].

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.). [^{3}H]B was obtained from Amersham International (Amersham, Bucks., U.K.). [^{3}H]A was prepared as described [4]. Briefly, placental extract was incubated in 12 nM

Abbreviations used: 11β-HSD 1, 11β-hydroxysteroid dehydrogenase type 1; A, 11-dehydrocorticosterone; B, corticosterone; E, cortisone; F, cortisol; DTT, dithiothreitol; IGF 1, insulin-like growth factor 1.

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[^{3}H]B and 1 mM NAD. [^{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, Witney, 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 μg/ml streptomycin and 200 mM L-glutamine. Cells were transferred into medium containing charcoal-stripped serum (prepared as described previously [25]) 24 h prior to hormone addition.

11β-HSD assays

Cells were plated at a density of 2×10^6/60-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 [^{3}H]steroid) was added 24 h after the addition of hormones. Generally, 200 μl aliquots of medium were taken 6 h after the addition of corticosteroid, and the [^{3}H]steroid extracted as described previously [21]. 11β-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 μl 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 [^{32}P]UTP-labelled, 125-base antisense β-actin cRNA was made by transcription using the pTRI-β-Actin-125 plasmid (supplied with the RPA II kit from Ambion) and T3 RNA polymerase. A [^{32}P]UTP-labelled, 579 bp 11β-HSD 1 antisense cRNA was made by transcription using T3 RNA polymerase with pBluescript SKII containing the rat 11β-HSD 1 cDNA (described in [19]) linearized at a *Sty*I site 579 bases from the 3′ end.

RNase protection was carried out using the RPA II kit supplied by Ambion. RNA (10 μg) was mixed with 2×10^6 c.p.m. of 11β-HSD 1 cRNA and 1×10^6 c.p.m. of β-actin cRNA, precipitated, resuspended in 20 μl 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 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 11β1-(−1800/+47)-luc, a luciferase construct containing a *Hin*dIII/*Pst*I fragment of the rat 11β-HSD 1 gene (−1800 to +47 relative to the major transcription start site) which was derived from clone λA described in [16] joined to the *Pst*I/*Hin*dIII region of the pUC 18 polylinker and inserted into the *Hin*dIII site in pSV0L.

Cells were plated at 5×10^5 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 mM L-glutamine. After 1 h, cells were transfected with 10 μg of plasmid DNA (5 μg of test plasmid, 5 μg 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 μl 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 μl of cell extract, 5 μl of 5 mM ATP, 100 μl of assay buffer [20 mM tricine, 1.07 nM $(MgCO_3)_4.Mg(OH)_2,5H_2O$, 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 μl of 1 mM luciferin. Light emission was measured over 10 s and relative light units (RLU)/μg 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 ± S.E.M.

RESULTS

11β-HSD activity and expression in 2S FAZA cells

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

Hormonal regulation of 11β-HSD in 2S FAZA cells

11β-HSD 1 enzyme activity and mRNA were measured in 2S FAZA cells treated for 24 h with 10 nM dexamethasone, 20 μg/ml insulin, 20 ng/ml insulin-like growth factor 1 (IGF 1), 10 μM forskolin or a combination of hormones, after which 11β-reductase activity was measured by the conversion of [^{3}H]A to [^{3}H]B in the medium over a period of 6 h (Table 1). Following 11β-HSD assay, cells were harvested and RNA encoding 11β-HSD 1 assayed by RNase protection analysis. Treatment with 10 nM dexamethasone led to a significant increase in 11β-reductase activity while 20 ng/ml IGF 1, 20 μg/ml insulin and 10 μM forskolin treatment all reduced 11β-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 11β-HSD 1 mRNA levels by RNase protection analysis demonstrated that 11β-HSD 1 mRNA levels

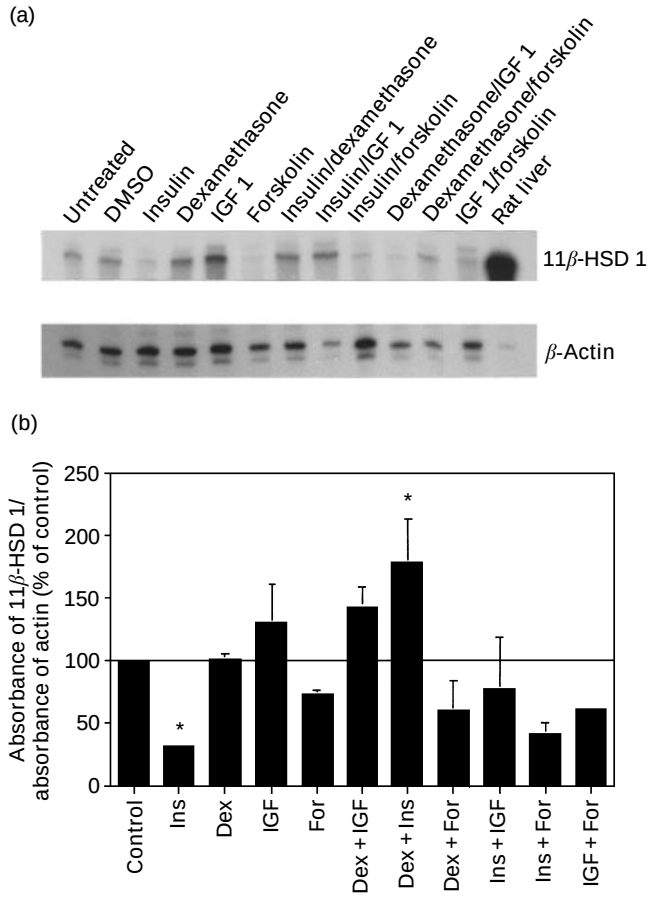

Figure 1 RNase protection analysis of 11β-HSD 1 mRNA levels in hormone-treated 2S FAZA cells

(**A**) Autoradiograph of RNase protection analysis of total RNA from hormone-treated 2S FAZA cells. Hormone treatment was as described for Table 1. A 10 μg sample of total RNA was hybridized with 1×10^6 c.p.m. of actin and 2×10^6 c.p.m. of 11β-HSD 1 radiolabelled cRNAs at 45 °C overnight. The autoradiograph was exposed for 2 h for the actin and 48 h for the 11β-HSD bands. The data are representative of three independent experiments. (**B**) Quantification of RNase protection assays carried out as described in (**A**) and expressed as the ratio between 11β-HSD 1 and actin. Data are expressed as a percentage of the control (untreated) level. The values are the mean $\pm$ S.E.M. derived from data from three independent experiments. Abbreviations: Ins, insulin; IGF, IGF 1; Dex, dexamethasone; For, forskolin. $^*P < 0.05$, compared with control.

Table 1 Effect of hormone treatments on 11β-HSD 1 enzyme activity in 2S FAZA cells in culture

Twenty-four hours prior to the addition of hormone, cells were plated in medium containing serum stripped of steroids and endogenous small peptides. Hormones used were dexamethasone (10 nM), insulin (20 μg/ml), IGF 1 (20 ng/ml) and forskolin (10 μM) either singly or in combination. Following the addition of hormone for 24 h, 11β-HSD activity in 2S FAZA cells was assayed by the conversion of [^{3}H]A added to the medium over a 6 h period. Activity is expressed as percentage of control $\pm$ S.E.M. Conversion by control cells was 16.4 $\pm$ 0.4%. Each value represents the mean of triplicate plates. The data are representative of three independent experiments. $^*P < 0.05$, compared with control.

Treatment	11β-HSD 1 activity (% of Control)
Control	100 $\pm$ 2
20 μg/ml (3.5 M) Insulin	51 $\pm$ 2*
10 nM Dexamethasone	192 $\pm$ 9*
20 ng/ml IGF 1	65 $\pm$ 2*
10 μM Forskolin	66 $\pm$ 7*
Insulin + dexamethasone	291 $\pm$ 4*
IGF 1 + dexamethasone	221 $\pm$ 4*
Forskolin + dexamethasone	142 $\pm$ 3*
Insulin + IGF 1	55 $\pm$ 4*
Insulin + forskolin	75 $\pm$ 7*
IGF 1 + forskolin	72 $\pm$ 7*

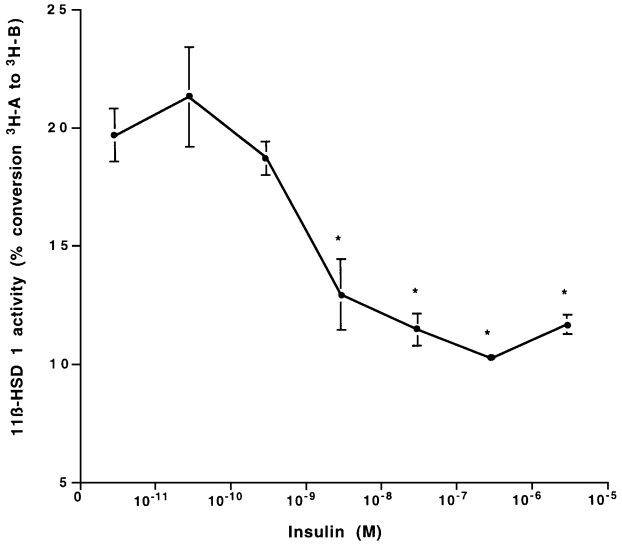

Figure 2 Concentration–response curve of 11β-HSD 1 activity with changing insulin concentration

For 24 h prior to the addition of insulin, cells were plated in medium containing serum stripped of steroids and endogenous small peptides. Following the addition of insulin for 24 h, 11β-HSD activity in 2S FAZA cells was assayed by the conversion of [^{3}H]A to [^{3}H]B in the medium over a 6 h period. Activity is expressed as percentage conversion $\pm$ S.E.M. of [^{3}H]A to [^{3}H]B. Each value represents the mean of triplicate plates. The data are representative of three independent experiments. $^*P < 0.01$, compared with 3×10^{-12} M.

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 *Hin*dIII-*Pst*I 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

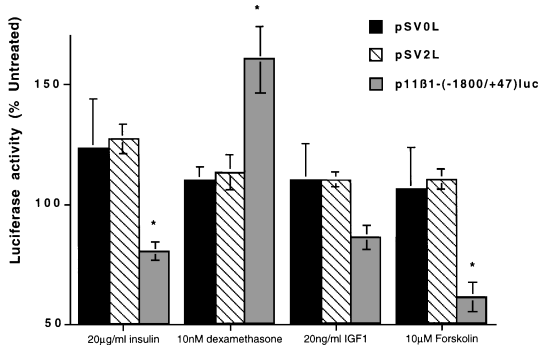

Figure 3 Effect of hormone treatments on transcription from the 11β-HSD 1 gene promoter

2S FAZA cells were transfected with p11β1-(−1800/+47)-luc (a luciferase reporter gene driven by the proximal promoter of 11β-HSD 1), pSV0L (a promoterless luciferase reporter gene) or pSV2L (a luciferase reporter gene driven by the SV40 promoter). Twenty-four hours after transfection, hormone was added and incubation continued for a further 24 h before harvesting for assay of luciferase activity. Results are expressed as the ratio of luciferase activity (RLU/μg of protein) in the absence of hormone to luciferase activity in the presence of hormone (fold induction ± S.E.M.). Typically, basal luciferase activity was 2.2 relative light units (RLU)/μg of protein for pSV0L, 850 RLU/μg of protein for pSV2L and 42 RLU/μg of protein for p11β-1(−1800/+47)-luc. All transfections were performed in triplicate, data are the means of at least three independent experiments. *$P < 0.05$ compared with pSV2L and pSV0L.

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-transcriptionally. 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 11β1-(−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 1 [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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