TNF-α enhances intracellular glucocorticoid availability

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Abstract For understanding the mechanism(s) relating inflammation to corticosteroid action, the effect of tumour necrosis factor-α (TNF-α) on 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the enzyme regulating access of 11β-hydroxycorticosteroids to receptors, was studied in LLC-PK$_1$ cells. We observed (i) NAD-dependent enzyme activity and mRNA for 11β-HSD2, but not 11β-HSD1, (ii) increasing 11β-HSD2 activity with increasing degree of differentiation and (iii) a concentration-dependent down-regulation by TNF-α, phorbol myristate acetate (PMA) or glucose of activity and mRNA of 11β-HSD2. The decrease of activity and mRNA by glucose and PMA, but not that by TNF-α, was abrogated by the protein kinase C inhibitor GF-109203X. The effect of TNF-α on 11β-HSD2 was reversed by inhibiting the mitogen-activated protein kinases ERK with PD-098050 and p38 by SB-202190, or by activating protein kinase A with forskolin. Overexpression of MEK1, an ERK activator, down-regulated the 11β-HSD2 activity. In conclusion, TNF-α decreases 11β-HSD2 activity and thereby enhances glucocorticoid access to glucocorticoid receptors to modulate the inflammatory response.

Key words: 11β-Hydroxysteroid dehydrogenase type 2; Tumor necrosis factor-α; Glucocorticoid

1. Introduction

During inflammation, glucocorticoids appear in higher extracellular concentrations, suggesting a modulating role in the stress response [1–3]. Two pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interleukin-1β, often exert synergistic effects, and have been shown to link inflammation with corticosteroid production by enhancing the secretion of corticotropin-releasing factor and thereby ultimately elevating corticosterone levels [2,3]. Intracellular glucocorticoid concentrations in target organs depend not only on the extracellular steroid concentrations, but in addition on the intracellular availability of these steroid hormones [1,4,5]. The intracellular access of steroids to receptors is regulated by the enzymes 11β-hydroxysteroid dehydrogenase types 1 and 2 (11β-HSD1 and 11β-HSD2), which interconvert biologically active 11β-hydroxysteroids and their inactive 11-keto congeners (reviewed in [6]).

Our group recently showed that TNF-α increases the expression and reductase activity of the [NADP(H)]-dependent enzyme 11β-HSD1 in non-endocrine cells [4]. As a consequence, 11-ketosteroids with low affinity for glucocorticoid receptors (GR) were converted into 11β-hydroxycorticosteroids, with high affinity for GR, and their biological activity was shown by inhibition of group II phospholipase A2 [4]. Thus, during inflammation, not only the hypothalamo–pituitary–adrenal axis is activated but, in addition, peripheral production of corticosteroids occurs by activation of 11β-HSD1.

11β-HSD2 requires NAD as a cofactor and shows predominantly oxidative activity by converting active 11β-hydroxy- into inactive 11-ketosteroids [6]. The 11β-HSD2 accounts for the selective access of aldosterone to the mineralocorticoid receptor (MR) by inactivating cortisol [6]. 11β-HSD2 is thus highly expressed in mineralocorticoid target tissues such as the renal cortical collecting duct and colon [6,7]. Absence or inhibition of 11β-HSD2 results in inappropriate access of glucocorticoids to epithelial MR with distal tubular sodium retention and increased blood pressure [8,9].

In the present investigation we focussed on the effect of TNF-α on 11β-HSD2 and found that TNF-α decreases the expression and activity of 11β-HSD2, an effect mediated by mitogen-activated protein (MAP) kinases and reversed by activating protein kinase A (PKA).

2. Materials and methods

2.1. Materials

Corticosterone, dehydrocorticosterone, glycyrrhetinic acid (GA) and phorbol 12-myristate 13-acetate (PMA) were from Sigma and NAD(H), NADP(H), deoxynucleotides, RNase inhibitor and avian myeloblastosis virus reverse transcriptase (AMV RT) from Roche Diagnostics. [1,2,6,7-^{3}H]Corticosterone (87 Ci/mmol) and [α-^{32}P]-dCTP (∼3000 Ci/mmol) were purchased from Amersham. Bicinchoninic acid protein assay kits were from Pierce. Thin layer chromatography plates (SIL G-25 UV$_{254}$) were from Macherey-Nagel (Oensingen, Switzerland). PD-98059, SB-202190, FTase inhibitor III and forskolin were from Calbiochem. Bisindolylmaleimide I hydrochloride salt (GF-109203X, abbreviated GFX) was from Alexis (San Diego, CA, USA) and Taq-Gold DNA polymerase from Perkin Elmer Cetus.

2.2. Cell cultures

LLC-PK$_1$ cells (porcine renal epithelial cells with characteristics of proximal and distal convoluted tubules) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 0.45% glucose, 10% foetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) [10]. For experiments cells were seeded at a density of 100 000 cells per well in 24-well culture plates (Becton Dickinson, Basel, Switzerland) or 400 000 cells per well in 6-well culture plates. Cells were stimulated after reaching confluence when abundant domes appeared, in the absence of serum. At the same time PD-098059 (50 μM), SB-202190 (10 μM), GFX (2 μM), PMA (100 nM), forskolin (10 μM)

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were added to the cultures for 24–48 h. The effect of TNF-α (Pharma Biotechnology, Hannover, Germany) on the viability of LLC-PK$_1$ cells was assessed by light microscopy, trypan blue dye exclusion, and release of lactate dehydrogenase (LDH) at the end of the incubations. Values for LDH release in the supernatant were calculated as percentage of total LDH release after freezing and thawing the respective cells.

2.3. Transient and stable overexpression of MEK1

pGEM-MEK (Ser218Asp; Ser222Asp) is a pGEM-7Zf(−) vector (Promega, Zurich, Switzerland) carrying a mouse MEK1 gene mutated at positions 218 and 222 from serine to aspartate (a gift from R.L. Erikson, Cambridge, MA, USA). This doubly mutated form of MEK1 has a four-fold higher ability to activate ERK1 compared to the wild-type form in intact cells [11]. For expression in LLC-PK$_1$ cells, the Asp718-*Nhe*I fragment from pGEM-MEK was subcloned into the Asp718-*Xba*I site of pcDNA3 (Invitrogen, Leek, The Netherlands) in the sense and antisense orientations. LLC-PK$_1$ cells were transfected with these pcDNA3-MEK1 plasmids or with the empty vector. Activity of stable clones was measured 2 days after reaching confluence in situ and total protein was determined as given below. For transient transfections 130 000 LLC-PK$_1$ cells were seeded into a 12-well plate. Cells were transfected the following day with 0.5 μg total DNA and 3 μl Fugene 6 (Roche Diagnostics, Basel, Switzerland) in a volume of 1 ml growth medium. Activity of 11β-HSD2 was assessed 48 h after transfection.

2.4. Reporter gene assay

We engineered LLC-PK$_1$ cells to carry the bacterial β-galactosidase gene (lacZ) under the control of the mouse mammary tumour virus (MMTV) promoter using a similar strategy as previously described by Satoh et al. [12]. The MMTV promoter contains essential glucocorticoid response elements. Thus the stably transfected LLC-PK$_1$ cell line expresses β-galactosidase when exposed to 11β-hydroxysteroids. For the assessment of the transactivation, LLC-PK$_1$ cells transfected with MMTV-lacZ were incubated after differentiation with 1 nM TNF-α with or without PD-098059 or the corresponding control solutions for 48 h. Thereafter the medium was replaced by charcoal-stripped DMEM (1 g/l glucose, 10% FCS) together with varying concentrations of corticosterone. After an additional 48 h the cells were subjected to the in situ β-galactosidase assay as described [12].

2.5. 11β-HSD type 1 and type 2 activity in whole cells and homogenates

11β-HSD2 activity was assessed by the conversion of corticosterone to 11-dehydrocorticosterone using medium with 5 nCi [^{3}H]corticosterone and 10 nM corticosterone at 37°C for 30 min and reductase activity using medium with 5 nCi [^{3}H]11-dehydrocorticosterone and 500 nM 11-dehydrocorticosterone [4]. 400 μM cofactor (NAD, NADH, NADPH) was added to homogenates only [10].

2.6. Reverse transcription and PCR

Total RNA was extracted from LLC-PK$_1$ cells following the guanidinium thiocyanate method. Concentration was determined by measuring the absorption at 260 nm and integrity was checked by loading 1 μg on a 1% formaldehyde agarose gel. 1 μg of each sample RNA was reverse transcribed into cDNAs using 1 U AMV RT and 10 pmol random hexamer primers according to the manufacturer's protocol.

Polymerase chain reaction was essentially performed as described previously [4]. Primers for 11β-HSD2 were: forward primer, 5′-TGCTGCAGATGGACCTGACCAA and reverse primer, 5′-TAGTAGTGGATGAAGTACATGAGC. These PCR primers were originally designed by Leckie et al. [10] to regions of 11β-HSD2 cDNA, which are conserved between the four species for which sequences of 11β-HSD2 were available (human, rat, rabbit, and sheep); these sequences are not found in other short-chain alcohol dehydrogenases. Primers for pig GAPDH (GenBank accession number U48832) were: forward primer, position 112–133 of the corresponding cDNA and reverse primer, position 685–704 of the corresponding cDNA.

2.7. Western blot analysis

Total protein (50 μg) was loaded on a denaturing 10% polyacrylamide gel. Prestained protein standards (Boehringer Mannheim) were used as markers. Electrophoresis and blotting on polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA) were performed as described in [4]. Detection of doubly phosphorylated ERK with a monoclonal antibody (New England Biolabs, Beverly, MA, USA) was performed according to the manufacturer's instructions. Detection was performed with the ECL Plus chemoluminescence detection kit (Amersham).

2.8. Analysis of data

Data are means (±S.D.). Statistical comparisons were performed using Welch *t*-test for independent means or ANOVA.

3. Results

3.1. Regulation of 11β-HSD2 activity by TNF-α, PMA and glucose

In LLC-PK$_1$ cells the oxidation of corticosterone to dehydrocorticosterone was determined in both cell lysates and whole cells. GA, a known inhibitor of 11β-HSD activity, reduced oxidative activity at 10 μM in whole cells and in cell extracts by more than 95% ($P < 0.001$, $n = 14$) [4,6]. Conversion of dehydrocorticosterone to corticosterone was measured in cell lysates and whole cells with 10 nM or 500 nM dehydrocorticosterone. No 11-oxoreductase activity was observed. Addition of 0.2% Triton X-100, an agent inhibiting 11β-HSD2 but not 11β-HSD1 activity [7], completely abolished conversion of corticosterone to dehydrocorticosterone.

To exclude competitive inhibition of 11β-HSD2 activity by TNF-α, glucose or PMA we tested similar concentrations of these substances in lysates of LLC-PK$_1$ cells; none decreased 11β-HSD2 activity.

The activity of 11β-HSD2 in LLC-PK$_1$ cells increased with increasing confluence and differentiation ($P < 0.0001$, Fig. 1). Addition of TNF-α (1 nM) down-regulated the activity of 11β-HSD2 in confluent cells ($P = 0.046$, Fig. 1) in media with or without serum. We checked three clones of LLC-

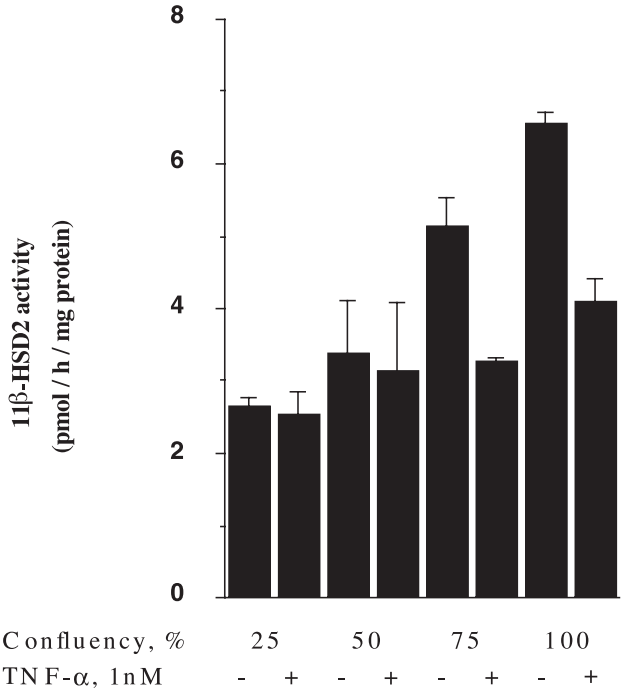

Fig. 1. Effect of confluence and TNF-α on 11β-HSD2 activity in LLC-PK$_1$ cells. The activity of 11β-HSD2 increased significantly with increasing confluence of cells with or without TNF-α. Data are given from a single study, repeated twice, performed in triplicate (mean ± S.D.).

PK_1 cells for 11β-HSD2 activity and found a range from 2 to 30 pmol/h/mg protein. In all clones TNF-α and PMA decreased the activity by about 50% after 48 h. For all subsequent experiments the clone with an activity of 8 ± 2 pmol/h/mg protein was used.

TNF-α down-regulated the activity of 11β-HSD2 dose-dependently (Fig. 2). This effect was not due to cell death, because after 48 h (i) more than 95% of cells were alive and (ii) an average of 15% ($n = 18$) increase of total protein was observed, reflecting proliferation of LLC-PK_1 cells [13]. For all subsequent experiments 1 nM of TNF-α was used.

Because 11β-HSD2 activity is differentiation-dependent, we tested whether activation of protein kinase C (PKC) affects 11β-HSD2 activity. PMA and glucose, two known PKC activators, decreased the activity of 11β-HSD2 by about 50% ($P < 0.0001$, Fig. 3). The effect of PMA and glucose but not of TNF-α was reversed with the PKC inhibitor GFX ($P = 0.0005$, Fig. 3A) [14,15]. TNF-α ($P < 0.0001$), PMA ($P = 0.002$) and high glucose ($P = 0.009$) decreased the 11β-HSD2 mRNA level in parallel with the activity of this enzyme (Fig. 3B). This effect was reversed by GFX only when the cells were incubated with PMA ($P < 0.0005$) or high glucose ($P = 0.0148$).

3.2. Role of MAP kinases in the regulation of 11β-HSD2

Because TNF-α is known to induce phosphorylation of ERK, a fact also observed in LLC-PK_1 cells 24 and 48 h after stimulation (Fig. 4A) the signal transduction pathway activated by TNF-α was explored. For this purpose we used the MAP kinase ERK inhibitor PD-098059, the MAP kinase p38 inhibitor SB-202190 [16,17] and the PKA activator forskolin. When confluent LLC-PK_1 cells were incubated with PD-098059 (50 μM), the activity of 11β-HSD2 increased ($P < 0.001$, Fig. 4); similar but less pronounced effects were seen with SB-202190 (10 μM) and forskolin (10 μM) ($P < 0.002$). The inhibitory effect of TNF-α on 11β-HSD2 was partly reversed by these inhibitors ($P = 0.01$) and forskolin ($P = 0.0003$) (Fig. 4). Down-regulation of 11β-HSD2 by

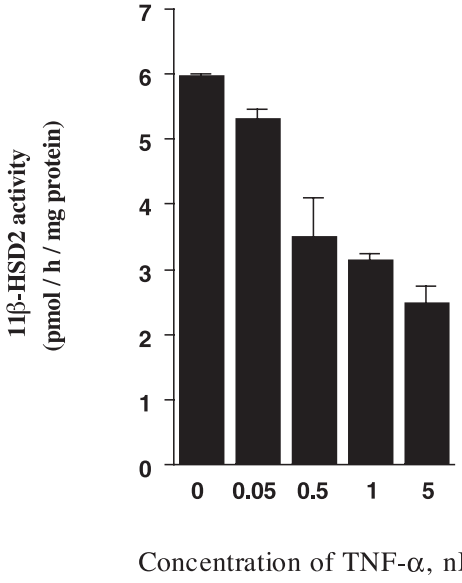

Fig. 2. Inhibition of 11β-HSD2 activity by TNF-α in LLC-PK_1 cells. Numbers as in Fig. 1.

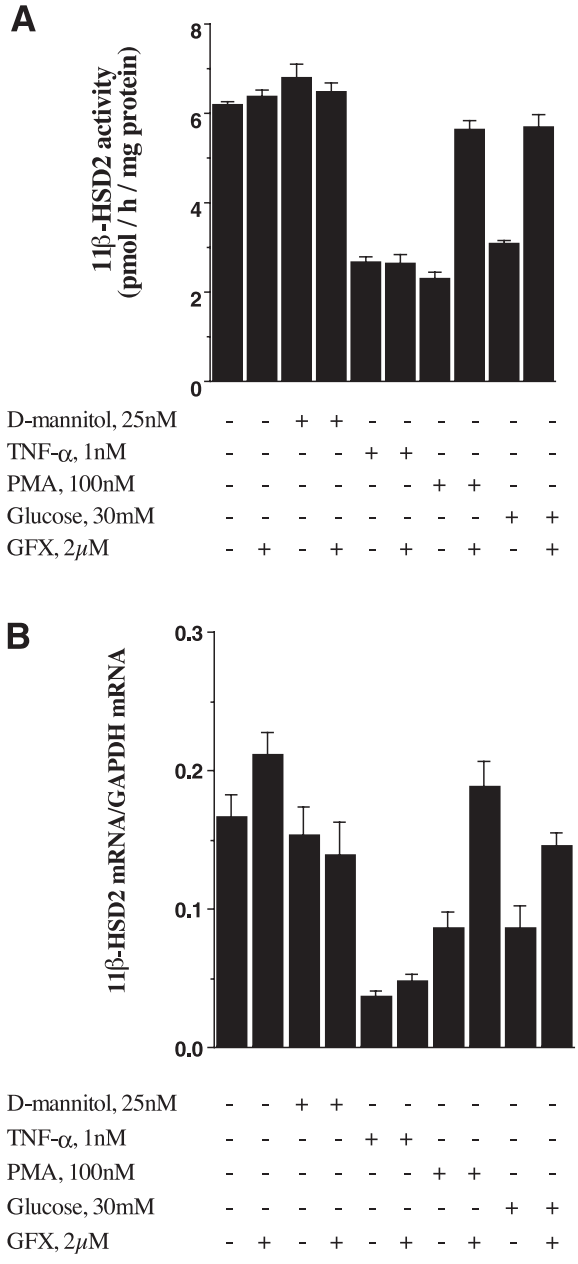

Fig. 3. Impact of TNF-α, PMA and high glucose concentrations with or without the PKC inhibitor GFX on the activity (A) and mRNA level (B) of 11β-HSD2 in LLC-PK_1 cells. TNF-α, PMA or high glucose concentrations reduced mRNA and activity of 11β-HSD2. GFX abrogated the inhibitory effect of PMA and high glucose on mRNA (A) and on 11β-HSD2 activity (B) but not of TNF-α. Numbers as in Fig. 1.

TNF-α was reversed with 50 μM of the ERK inhibitor PD-098059 (data not shown). In line with these inhibition studies transient overexpression of MEK1 reduced the activity of 11β-HSD2 ($P = 0.007$) (Fig. 5). In addition to the transient overexpression studies three different stably transfected clones overexpressing MEK1 were analysed. The basal activities of 11β-HSD2 of MEK1-expressing clones in relation to their corresponding MEK1 antisense-expressing cells (= 100%) were 53.5 ± 20.6% (range 29.8–67.7% with a median of 63.1%). There was no difference between antisense- and empty vector-transfected cells in terms of 11β-HSD2 activity.

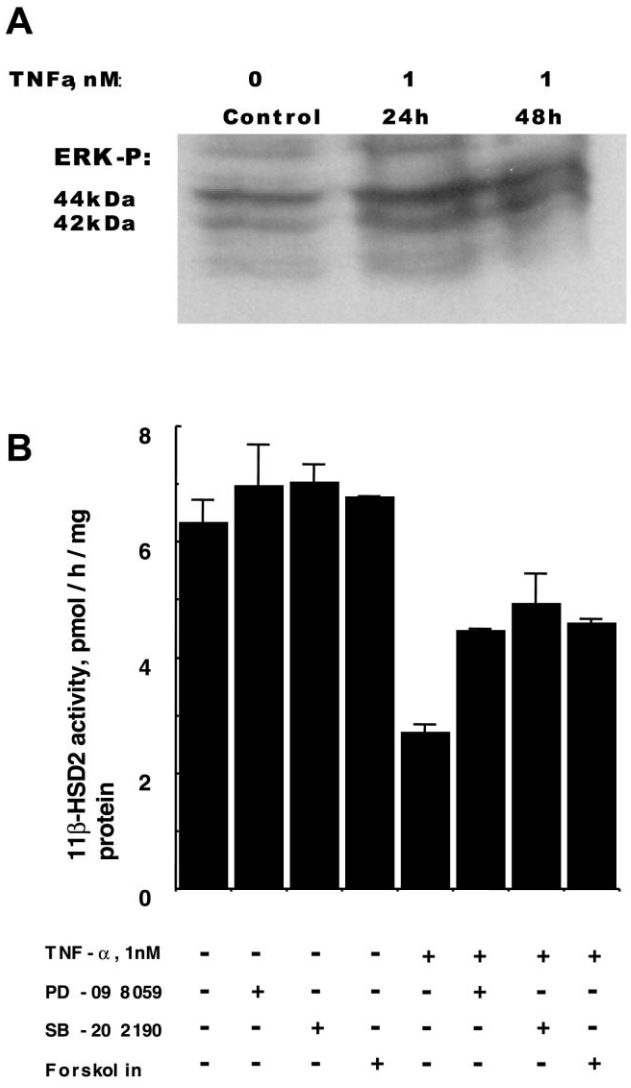

Fig. 4. A: Western blot of cell extracts of phosphorylated ERK (42 and 44 kDa) of LLC-PK$_1$ cells stably transfected with MEK1. Cells were incubated without or with 1 nM TNF-α for 24 and 48 h. 50 μg was loaded per lane. B: Effect of inhibition of the MAP kinases ERK by PD-098059 and p38 by SB-202190 and of the activation of PKA by forskolin in LLC-PK$_1$ cells in the presence and absence of TNF-α. The mean (±S.D.) of two independent experiments performed in triplicate is given.

3.3. *TNF-α down-regulates activity of 11β-HSD2 and increases the biological effect of glucocorticoids*

To clarify whether the down-regulated activity of 11β-HSD2 by TNF-α increases the biological effect of glucocorticoids we engineered LLC-PK$_1$ cells with a stably inserted bacterial β-galactosidase gene controlled by the MMTV promoter which contains several steroid response elements. This cell line therefore expresses β-galactosidase only when active glucocorticoids are present. Indeed, TNF-α enhanced the 11β-hydroxysteroid corticosterone effect, an effect reversed by inhibiting ERK with PD-098059 (Fig. 6). When GA was added together with corticosterone the steroid action was increased, indicating an important role of 11β-HSD2 in modulating steroid-induced gene expression.

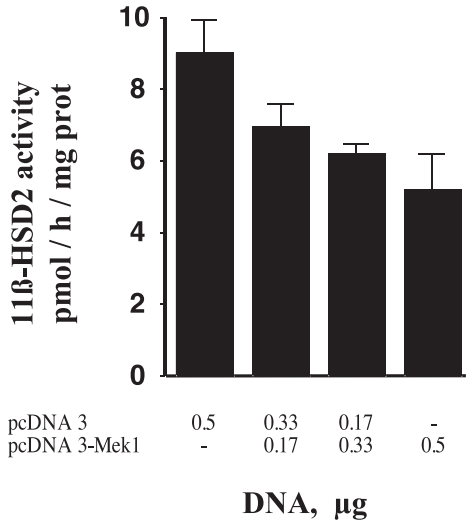

Fig. 5. Transient overexpression of MEK1 decreases 11β-HSD2 activity. In each well 0.5 μg of total DNA was transfected. The relative amount of pcDNA3-MEK1 plasmid increases from left to right resulting in a dose-dependent decrease of 11β-HSD2 activity. The results from a single study performed in triplicate are given; the experiment was repeated twice with the same results.

4. Discussion

Confluent LLC-PK$_1$ cells differentiate [18]. Therefore we analysed the activity of 11β-HSD2 as a function of the confluence stage and showed that increasing confluence increased the 11β-HSD2 activity (Fig. 1). A comparable stage and differentiation dependence has been reported for the Na^+/glucose cotransporter, the formation of tight junctions, the transepithelial salt or water transport and brush border enzymes in LLC-PK$_1$ cells [18,19]. This present observation of differentiation-dependent 11β-HSD2 induction in vitro is relevant for understanding in vivo situations. Several groups have observed complex developmental patterns in vivo for activity

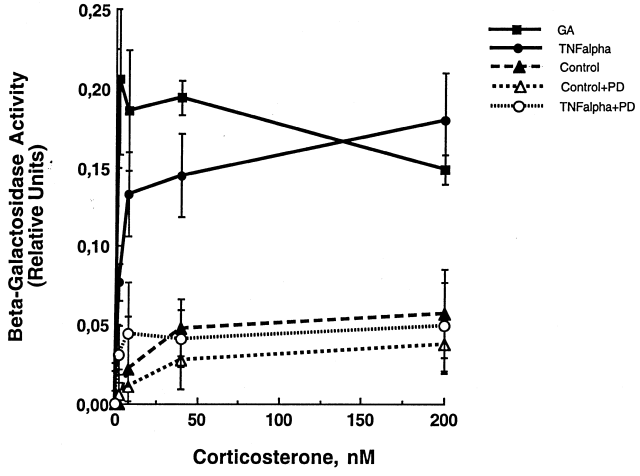

Fig. 6. Influence of TNF-α with and without inhibition of ERK by PD-098059 (PD) on glucocorticoid-dependent gene expression of β-galactosidase. Addition of TNF-α increased corticosterone-induced β-galactosidase activity. Inhibition of ERK by PD-098059 reversed this effect. Inhibition of 11β-HSD2 with GA (10 μM) elevated the steroid-induced β-galactosidase activity. Numbers as in Fig. 5.

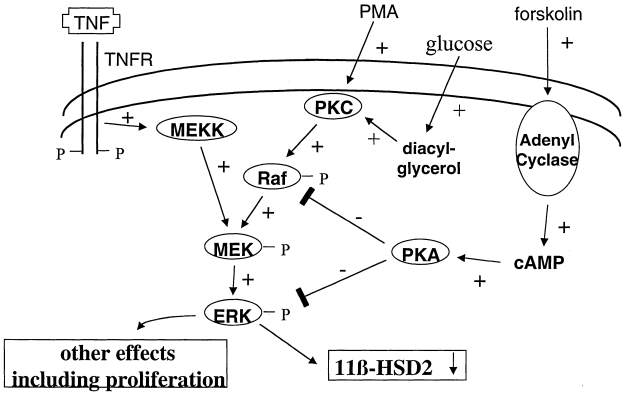

Fig. 7. A simplified model of 11β-HSD2 regulation.

and/or 11β-HSD1 mRNA levels and/or 11β-HSD2 mRNA levels in foetal or early postnatal tissue [20,21]. Down-regulation of 11β-HSD2 means that the corresponding cells are exposed to increased intracellular concentrations of 11β-hydroxysteroids, and up-regulation of 11β-HSD2 has the opposite effect. Thus each individual cell regulates the exposure to glucocorticosteroids differentially according to its developmental stage.

At least two different signal transduction pathways are involved in the regulation of 11β-HSD2 (Fig. 7). One pathway involves activation of PKC and MAP kinases, the second the activation of MAP kinases by a PKC-independent pathway. Previous studies have shown that TNF-α and PKC cause downstream activation of MAP kinases [22,23]; three lines of evidence in the present investigation support the relevance of PKC in the regulation of 11β-HSD2 activity. First, the inverse relationship between the activity of PKC and 11β-HSD2 during cellular growth is indirect and correlative evidence; PKC activity is high during cellular growth and low at confluence when the cell cycle is arrested [18]; in contrast, the activity of 11β-HSD2 is low during growth and high at confluence, when LLC-PK_1 cells differentiate and stop cycling (Fig. 1). Secondly, PMA reduces the activity of 11β-HSD2, an effect abrogated by the PKC inhibitor GFX (Fig. 3). Third, high glucose concentrations diminish the activity of 11β-HSD2 in LLC-PK_1 cells; high glucose concentrations are known to exhibit a similar effect on PKC as PMA [14]. The glucose-induced decline in 11β-HSD2 activity (Fig. 3) is in accordance with the reduced 11β-HSD2 observed in rats treated with streptozotocin [24].

Both MAP kinases ERK and p38 are involved in the TNF-α-induced reduction of 11β-HSD2 expression. Inhibition of ERK in confluent LLC-PK_1 cells leads to an increase in 11β-HSD2 activity which may be due to undifferentiated, dividing cells in culture with active ERK (Fig. 4) [25]. TNF-α induces proliferation and dedifferentiation of LLC-PK_1 cells, an effect paralleled by down-regulation of tissue-specific genes such as 11β-HSD2 [19]. The observation that LLC-PK_1 cells are responsive to TNF-α, an effect partially reversed by forskolin, is in line with the report that the transepithelial resistance response of LLC-PK_1 cells to TNF-α can be abrogated by forskolin [26]. Forskolin enhanced 11β-HSD2 activity, a finding in accordance with the up-regulation of 11β-HSD2 by forskolin in JEG-3 cells [27], a human choriocarcinoma cell line. The mechanism of action of forskolin in this model is open to speculation. Forskolin induces cAMP-dependent PKA. PKA is an important physiological inhibitor of the MAP kinase cascade and cell proliferation by inhibiting Raf-1 [28] and/or by inducing phosphatase activity [29]. Thus, it is conceivable that forskolin reduced the effect of TNF-α on 11β-HSD2 activity by inhibition of MAP kinase activation. Alternatively, forskolin might induce other transcription factors, such as CREB.

The biological relevance of enhanced access of glucocorticoids to receptors by TNF-α-induced down-regulation of 11β-HSD2 activity was qualitatively assessed in a reporter gene assay (Fig. 7), and the effect of TNF-α reversed by inhibiting the ERK-dependent signal transduction pathway. The inhibitory effect of TNF-α on 11β-HSD2 activity was more pronounced in this reporter gene assay than one would predict on the basis of the relative inhibition of 11β-HSD2 in untransfected LLC-PK_1 cells by TNF-α and GA, suggesting that factors in addition to ligand metabolism may account for the modulation of the corticosteroid-induced β-galactosidase expression by TNF-α observed in transfected cells. One possible explanation is the recently described enhanced transcriptional activity of steroid receptors by TNF-α [30].

Zipser et al. [31] observed low aldosterone despite high renin levels in septic patients with appropriately elevated plasma cortisol concentrations. The most likely mechanism for this hyperreninaemic hypoaldosteronism is TNF-α-mediated inhibition of angiotensin II- and ACTH-induced expression of aldosterone synthase [32,33]. Thus aldosterone does not explain the clinically beneficial increased renal sodium retention which is seen as part of the complex haemodynamic response in septic patients. The present observation, however, that TNF-α down-regulates transcription and activity of 11β-HSD2, does. Under normal conditions 11β-HSD2 protects mineralocorticoid receptors from glucocorticoids. When the activity of 11β-HSD2 is reduced, as it is in the presence of TNF-α, glucocorticoids act as mineralocorticoids and enhance renal sodium retention [6,8,9,34].

In conclusion, TNF-α increases the formation of biologically active glucocorticoids at the site of action by enhancing the reductase activity of 11β-HSD1 as previously shown [4], and by inhibiting the oxidative activity of 11β-HSD2 as demonstrated in the present investigation. This differential regulation of the expression of 11β-HSD 1 [4] and 11β-HSD2 by TNF-α might contribute not only to overcoming unwanted effects in inflammatory stress as discussed recently [4], but also to enhancing renal sodium retention by inappropriate access of glucocorticoids to epithelial MR.

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