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Reduced 11β-hydroxysteroid dehydrogenase activity in experimental nephrotic syndrome

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Abstract

Background. The disease state of the nephrotic syndrome is characterized by abnormal renal sodium retention that cannot be completely explained by a secondary hyperaldosteronism for the following reasons. Firstly, in rats an enhanced sodium retention is observed before proteinuria with intravascular volume depletion occurs. Secondly, in patients with the nephrotic syndrome, volume expansion with hypertension has been reported despite suppression of the renin-aldosterone system. Therefore, another mechanism for sodium retention must be postulated for this disease state. We hypothesize that this mechanism is a reduced 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) activity, a phenomenon known to cause enhanced access of cortisol or corticosterone to the mineralocorticoid receptor.

Methods. We assessed the 11β-HSD activity by measuring the urinary ratio of tetrahydrocorticosterone (THB) plus 5α-tetrahydrocorticosterone (5α-THB) to 11-dehydro-tetrahydrocorticosterone (THA) by gas chromatography–mass spectrometry in rats with puromycin aminonucleoside (PAN)-induced proteinuria and with adriamycin nephrosis. Furthermore, the plasma ratios of corticosterone to 11-dehydrocorticosterone were measured.

Results. The urinary ratio of (THB + 5α-THB)/THA increased in all animals following injection of PAN or adriamycin, indicating a reduced activity of 11β-HSD. The reduced activity of 11β-HSD was confirmed by an increased plasma ratio of corticosterone to 11-dehydrocorticosterone. The changes in the glucocorticoid metabolite ratios were already present before significant proteinuria appeared.

Conclusion. PAN- or adriamycin-treated rats develop proteinuria with a reduced activity of 11β-HSD, a mechanism contributing to the abnormal sodium retention in nephrotic syndrome.

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Introduction

11β-Hydroxysteroid dehydrogenase (11β-HSD) enzymes catalyse the interconversion of endogenous or exogenous biologically active 11β-hydroxy-glucocorticosteroids (cortisol, corticosterone or prednisolone) into inactive keto-glucocorticosteroids (cortisone, 11-dehydrocorticosterone or prednisone, respectively) [1–4]. Currently two isoenzymes, 11β-HSD1 and 11β-HSD2 accounting for 11β-HSD activity have been cloned and characterized. The enzymes only share a 14% homology and have different physiological roles, regulation, and tissue distribution. 11β-HSD1 acts predominantly as a reductase *in vivo*, is localized in the endoplasmic reticulum membrane with a luminal orientation of the catalytic domain, is NADP-dependent, has a K_m in the micromolar range, and is expressed in most tissues. Its biological relevance is thought to be as the catalyst for the reactivation of cortisone to cortisol, and by that mechanism might regulate glucocorticosteroid access to glucocorticosteroid receptors [4–7]. 11β-HSD2 on the other hand displays 11β-oxidase activity, is localized in the endoplasmatic reticulum membrane with a cytoplasmatic orientation of the catalytic domain, is NAD-dependent, has a nanomolar K_m and is preferentially found in tissues expressing mineralocorticoid receptors, including the cortical collecting duct of the kidney [4,7–10]. The pivotal role of 11β-HSD2 in excluding endogenous glucocorticoids from the mineralocorticoid receptor is now widely accepted.

A reduced activity of 11β-HSD2 causes renal sodium retention [11]. There is growing evidence that such a down-regulation of 11β-HSD2 accounts, at least partly, for abnormal sodium retention in disease states such as

liver cirrhosis [12,13]. Furthermore, we recently showed that patients with the nephrotic syndrome have a reduced urinary ratio of tetrahydrocortisol (THF) plus 5α-tetrahydrocortisol (5α-THF) to 11-dehydrotetrahydrocortisone (THE), suggesting a diminished activity of 11β-HSD2 [14]. The 29 nephrotic patients investigated were treated with a variety of different drugs that might interfere with 11β-HSD2 activity, and exhibited seven different underlying disease states causing the nephrotic syndrome. Therefore, to show unambiguously that sodium retention in proteinuric states is associated with a decline in 11β-HSD2 activity, we induced proteinuria by puromycin aminonucleoside (PAN) or adriamycin in rats. We assessed the activity of 11β-HSD2 by measuring the urinary ratio of tetrahydrocorticosterone (THB) plus 5α-tetrahydrocorticosterone (5α-THB) to 11-dehydrotetrahydrocorticosterone (THA), and the plasma ratio of corticosterone to 11-dehydrocorticosterone.

Materials and methods

Supplies

Corticosterone, 11-dehydrocorticosterone, PAN and adriamycin were purchased from Sigma Chemical Co. (Buchs, Switzerland). Bicinchoninic acid protein assay reagent (BCA) was received from Pierce Chemical Co. (Rockford, IL, USA). The 5α-androstane-3α, 17α-diol, stigmasterol and cholesteryl butyrate were purchased from Steraloids (Wilton, NH, USA).

Experimental animals

The protocol was approved by the ethics committee at our institution. Male Wistar rats, weighing 200–230 g were kept in a temperature-, humidity- and light-controlled room (12-h light, 12-h dark cycle) and maintained on a normal chow diet without fluid restriction. Animals were kept in metabolic cages for urine collection. After an adaptation period of 4 days, the rats received either a single IP dose of PAN (15 mg/100g body weight in 1 ml of NaCl 0.9%) for the induction of proteinuria, or the solvent only (1 ml of NaCl 0.9%). Adriamycin nephrosis was induced by a single dose of 7.5 mg/kg body weight, freshly prepared and diluted to 0.75 mg/kg body weight into the tail vein [15–18].

Steroid analysis by gas chromatography–mass spectrometry

Analysis of corticosterone and 11-dehydrocorticosterone in plasma. For analysing corticosterone and 11-dehydrocorticosterone, 100 ng of 11α-tetrahydrocortisol was added to 1 ml of plasma as an internal standard, and the sample was extracted with 10 ml of dichlormethane on a rotator. After centrifugation at 3000× *g* for 5 min and separation of the phases, the organic layer (containing the unconjugated steroids) was evaporated under a stream of nitrogen at room temperature. The sample was derivatized to form the methyloxime-trimethylsilyl ethers. The excess derivatization reagent was removed by gel filtration on a Lipidex-5000 column. As an internal standard, 100 ng of 11α-tetrahydrocortisol was added to the water phase (containing the conjugated steroids). Plasma proteins were precipitated with 5 ml of methanol. After evaporation of the solvent, the sample was reconstituted in 0.1 M acetate buffer and hydrolyzed with 12.5 mg of powdered *Helix pomatia* enzyme (Sigma) and 12.5 µl of β-glucuronidase/arylsulphatase (Roche, Basel, Switzerland) at 55°C for 3 h. The hydrolysis mixture was extracted with a Sep Pak C18 column, and the resulting free steroids were derivatized to form the methyloxim-trimethylsilyl ethers. The excess derivatization reagent was removed by gel filtration on a Lipidex-5000 column.

Samples were analysed by gas chromatography–mass spectrometry using a Hewlett–Packard gas chromatograph 6890 equipped with a mass selected detector 5973 by selective ion monitoring. One characteristic ion was chosen for each of the compounds being measured. The ion number (m/z) 548, 474 and 652, were chosen for corticosterone, 11-dehydrocorticosterone and 11α-tetrahydrocortisol, respectively. Calibration lines were established over the range of 5 ng/ml to 100 ng/ml. Correlation coefficients were >0.98. Coefficients of variation for intra- and inter-day analysis were $<15\%$.

Analysis of steroid metabolites in urine. Urine sample preparation consists of pre-extraction, enzymatic hydrolysis, extraction from the hydrolysis mixture, derivatization and gel filtration [19]. Briefly, 2.5 µg of medroxyprogesterone was added to 1.5 ml of urine as recovery standard. The sample was extracted on a Sep-Pak C 18 column, dried, reconstituted in 0.1 M acetate buffer, adjusted to pH 4.6 and hydrolysed with powdered *Helix pomatia* enzyme (12.5 mg; Sigma) and 12.5 µl of β-glucuronidase/arylsulphatase liquid enzyme (Roche, Basel, Switzerland). The resulting free steroids were extracted on a Sep-Pak C 18 cartridge. The mixture of internal standards (5α-androstane-3α, 17α-diol, stigmasterol, cholesteryl butyrate, 2.5 µg each) was added to this extract, and the sample was derivatized to form the methyloxime-trimethylsilyl ethers. One characteristic ion was chosen for each compound being measured. The ion number (m/z) 564, was monitored for THB and 5α-THB, and 490, for THA. A temperature-programmed run from 210 to 265°C over 35 min was chosen. As a calibration standard, a defined steroid mixture of the measured steroid metabolites was analysed. The 'ion peak' abundance was quantified against the internal standard stigmasterol. Using this method the following metabolites were determined in urine: THB, 5α-THB and THA.

Analysis of data

Data were analysed by Student's paired two-tailed *t*-test. Results are given as mean ± SEM.

Results

The urinary ratio of (THB + 5α-THB)/THA increased from day 0 to day 1 in all animals. Accordingly, the mean ratios increased from 1.41 ± 0.12 to 2.25 ± 0.25 ($P < 0.01$) following the injection of PAN, and from 1.48 ± 0.11 to 2.95 ± 0.30 ($P < 0.05$) following the

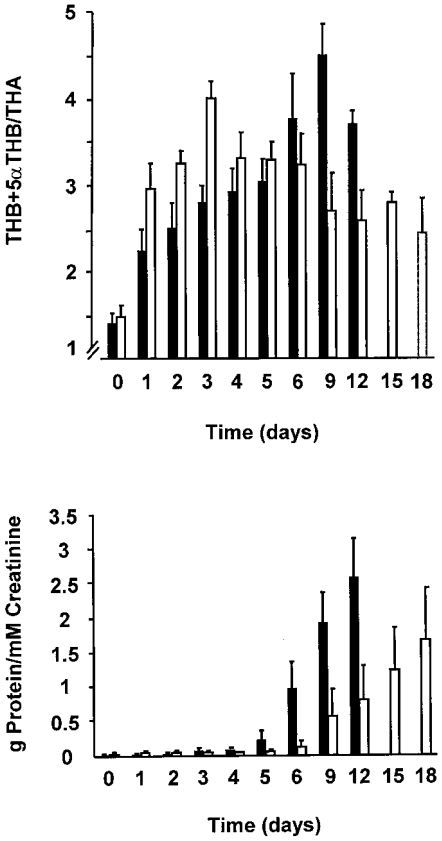

Fig. 1. Effect of PAN- (black bars) and adriamycin- (open bars) induced nephrotic syndrome on the ratio of corticosterone metabolites (THB + 5α-THB)/THA) and protein excretion in urine. Each column represents the mean ± SEM from six animals. The measurements were performed in 24-h urine collections. The ratio of corticosterone metabolites had already increased during the initial 24 h in all animals.

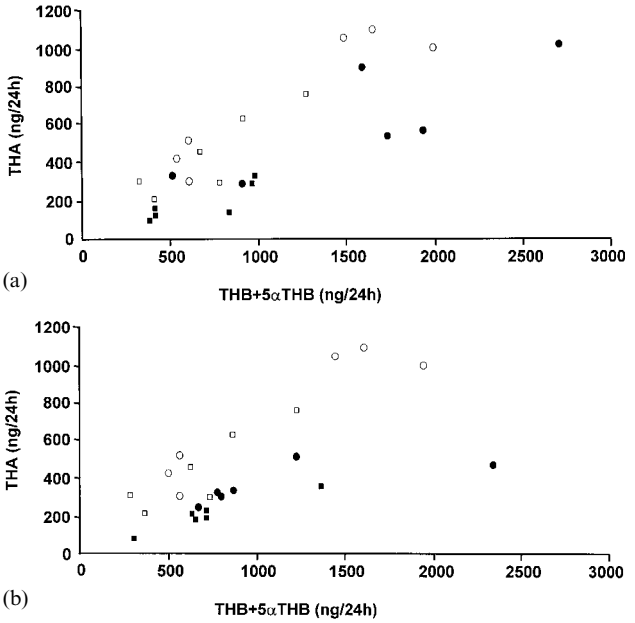

Fig. 2. (**a**) Urinary excretion of THA as a function of THB + 5α-THB. Open signs (○ and □) represent the values from day 0 before the administration of PAN or adriamycin, and closed signs (● and ■) represent the values at the day 3. The PAN-treated animals are represented by squares (□ and ■) and the adriamycin-treated animals by circles (○ and ●). (**b**) Urinary excretion of THA as a function of THB + 5α-THB. Open signs (○ and □) represent the values from day 0 before the administration of PAN or adriamycin, closed signs (● and ■) represent the values at the end of the experiment when proteinuria was maximal, i.e. day 12 for the PAN-treated animals (squares: □ and ■) and day 18 for the adriamycin treated animals (circles: ○ and ●).

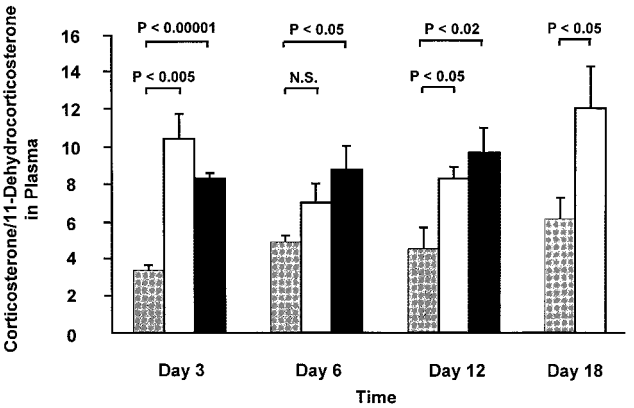

Fig. 3. Plasma ratio of corticosterone/11-dehydrocosticosterone. Grey bars represent control animals, white bars adriamycin-treated animals and black bars PAN-treated animals. Each column represents the mean ± SEM from six animals.

injection of adriamycin (Figure 1). The increased ratios of (THB + 5α-THB)/THA were attributable to a decreased urinary excretion of THA in proteinuric animals. This effect is best shown by comparing the 24 h urinary excretion of THA as a function of THB + 5α-THB. To illustrate, the results from days 0 and 3 (Figure 2a) and from days 12 and 18 (Figure 2b) in the PAN- and adriamycin-treated animals are shown. Analysis of plasma corticosterone/11-dehydrocorticosterone ratios revealed an increase in both animal models (Figure 3).

Compared with the increase in the urinary steroid metabolite ratio, the urinary protein excretion occurred later (Figure 1). On day 6 in the PAN group, and on day 9 in the adriamycin group, all six animals in each group had increased urinary protein excretion rates. The urinary sodium/potassium ratio declined from 0.61 ± 0.05 on day 0 to 0.43 ± 0.03 on day 2 ($P < 0.05$) in the PAN-treated animals, and from 0.48 ± 0.03 on day 0 to 0.30 ± 0.04 on day 4 ($P < 0.02$) in the adriamycin-treated animals, indicating an enhanced mineralocorticoid effect.

Discussion

PAN and adriamycin induce proteinuria with avid renal salt retention and structural changes in the glomerulus and renal tubules, which is characteristic of minimal change glomerulopathy [15–17,20,21]. Therefore, these two models of experimental nephrotic

syndrome in rats are widely used to investigate the mechanisms accounting for the nephrotic disease state. The time course for the appearance of proteinuria in our groups of animals given either PAN or adriamycin is in line with that observed in the literature [16,20,22]. Interestingly, in both models, the increase in the urinary ratio of (THB + 5α-THB)/THA was present before a significant urinary excretion of protein was observed (Figure 1). This observation might account for the hitherto unexplained finding that sodium retention occurs before the onset of proteinuria in PAN- and adriamycin-treated rats [15,16,22]. Such a dissociation between renal sodium retention and proteinuria is not restricted to these two models but has also been described in other situations, such as Heymann nephritis or serum sickness [23].

The dissociation between the appearance of proteinuria and renal sodium retention is one of the arguments forwarded by the critics of the traditional view of renal sodium retention in nephrotic patients. Traditionally, the increased renal sodium retention in nephrotic patients has been attributed to a secondary hyperaldosteronism, as a consequence of renal hypoperfusion related to fluid sequestration [24]. This concept has been questioned, firstly, by the results from the above mentioned experimental models [16,20–23] and, secondly, by the observation that adrenalectomized rats injected with PAN develop a full-blown nephrotic syndrome [22]. Therefore, another mechanism for sodium retention in nephrotic subjects has to be postulated. An aldosterone-independent candidate mechanism is a reduced activity of 11β-HSD2, a characteristic which provides promiscuous access of glucocorticoids, such as cortisol or corticosterone, to the mineralocorticoid receptor [1,3,4]. The present observation of an increased urinary ratio of (THB + 5α-THB)/THA and an increased plasma ratio of corticosterone/11-dehydrocorticosterone in rats with nephrotic syndrome, together with our previous finding of an increased urinary ratio of (THF + 5α-THF)/THE in humans with proteinuria [4], strongly supports the contention of a reduced renal 11β-HSD2 activity in this disease state.

Deschenes *et al.* [16] previously performed meticulous salt balance studies in rats with PAN- and adriamycin-induced nephrotic syndrome. They observed an early decrease in sodium excretion in PAN-treated animals starting on day 2, an observation in line with the decreased urinary sodium/potassium ratio found in our rats treated with the same agent. Similarly, the effect was less pronounced in both studies when rats were given adriamycin [16]. These effects paralleled the decline in the apparent activity of 11β-HSD2, as assessed by the steroid metabolites. In both studies, the changes in the urinary electrolyte excretion preceded the appearance of proteinuria.

The increased urinary ratio of (THB + 5α-THB)/THA presumably reflects a reduced activity of 11β-HSD2 and not of total 11β-HSD (i.e. 11β-HSD1 plus 11β-HSD2) activity, because in 11β-HSD1 knockout mice, which have a lack of functioning 11β-HSD1 enzyme, the urinary ratio of (THB + 5α-THB)/THA is not altered [25]. It has been proposed that the ratio of urinary corticosterone/dehydrocorticosterone in rodents, or cortisol/cortisone in humans, may be more suitable for indicating the activity of renal 11β-HSD2 [26–29]. Recently, Ferrari *et al.* [30], in an in depth analysis in human subjects with or without salt-sensitivity, have shown that the cortisol/cortisone ratio does not appear to be more sensitive for detecting changes in 11β-HSD2 activity than (THF + 5α-THF)/THE, which is the analogue of (THB + 5α-THB)/THA in rodents. Because of this controversy, we measured the corticosterone/11-dehydrocorticosterone ratios as well as the (THB + 5α-THB)/THA ratios, in both animal models. Both measures point to a decreased 11β-HSD2 activity. Moore *et al.* [31] studied extrarenal localization of 11β-HSD2 expression in the mouse at the level of gene transcription, and found that 11β-HSD2 was expressed in the colon, small intestine, stomach and epididymus, in addition to renal tissue. Furthermore, expression of 11β-HSD2 has been found in human vascular smooth-muscle cells [32]. It is not known if inhibition of extra-renal 11β-HSD2 alters the urinary (THB + 5α-THB)/THA ratio in rats. In patients without renal 11β-HSD2 activity, inhibition of extra-renal activity by glycyrrhetinic acid resulted in an increased plasma ratio of cortisol/cortisone [33]. However, the relevance of this finding for the urinary (THB + 5α-THB)/THA ratio is not known.

The mechanism for the early decline in the apparent 11β-HSD2 activity, assessed *in vivo* by measuring urinary glucocorticosteroid metabolites, in our PAN- and adriamycin-treated rats is unknown (Figure 1). Two mechanisms have to be considered. Firstly, it is conceivable that the xenobiotics directly caused the decline of the apparent activity of 11β-HSD2. This is unlikely to be the case because, when we incubated cells expressing 11β-HSD2, no inhibitory effect of PAN or adriamycin was observed (unpublished observation). Secondly, a reduced 11β-HSD2 activity *in vivo* can occur in the presence of inhibitors. Morris *et al.* [34,35] observed glycyrrhetinic acid like factors (GALF) in urine from patients with hypertension or heart failure, indicating that these inhibitors of 11β-HSD activity are water soluble. In patients with cholestasis, bile acids reduce 11β-HSD2 activity which results in MR activation by cortisol [36]. Future studies must be carried out to define the GALF or other inhibitors that are presumably present in the nephrotic syndrome.

Ichikawa *et al.* [21], in a seminal investigation using a unilateral model of PAN-induced albuminuria and micropuncture methodology, demonstrated that the enhanced sodium retention was only present in the proteinuric kidney and was clearly localized downstream of the distal tubule. Later on, in collaboration with H. Favre [20,22], one of us showed that the Na,K-ATPase activity is significantly enhanced in the cortical collecting duct (CCT) from rats with PAN-induced nephrotic syndrome. Vasopressin is known to stimulate Na,K-ATPase activity in the CCT [37]. Recently, Deschênes and Doucet [16] showed that

ADH cannot account for the enhanced Na,K-ATPase activity in ADH-deficient Brattleboro rats treated with PAN. Thus, at the present time the best established factors for up-regulating the Na,K-ATPase in the CCT remain mineralo- or gluco-corticosteroids [38,39]. A decreased activity of 11β-HSD has been shown to enhance the intracellular availability of corticosteroids to the cognate receptor [4,6]. Furthermore, Biller *et al.* [11] recently showed direct *in vivo* evidence for a reduction in fractional sodium excretion during inhibition of 11β-HSD2 in the collecting duct. Therefore, the present observation of increases in (THB + 5α-THB)/THA ratios in urine, and in corticosterone/11-dehydrocorticosterone ratios in plasma, suggests that a reduced activity of 11β-HSD2 contributes to the abnormal sodium retention in the animal models investigated. This observation is supported by the decrease in the urinary sodium/potassium ratio shortly after the administration of the xenobiotics for the induction of the nephrotic syndrome, indicating increased mineralocorticoid activity.

In conclusion, the previous observations in patients with the nephrotic syndrome [14] and the present study in two well established animal models indicate that the activity of 11β-HSD2, assessed by the urinary (THB + 5α-THB)/THA ratio, is reduced in nephrotic syndrome. These observations suggest that endogenous glucocorticoids exhibit a mineralocorticoid effect and contribute to abnormal sodium retention in nephrotic syndrome.

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