Intestinal inflammation modulates expression of 11β-hydroxysteroid dehydrogenase in murine gut

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Abstract

The effect of glucocorticoids is controlled at the pre-receptor level by the activity of 11β-hydroxysteroid dehydrogenase (11HSD). The isoform 11HSD1 is an $NADP^+$-dependent oxidoreductase, usually reductase, that amplifies the action of glucocorticoids due to reduction of the biologically inactive 11-oxo derivatives cortisone and 11-dehydrocorticosterone to cortisol and corticosterone. The NAD^+-dependent isoform (11HSD2) is an oxidase that restrains the effect of hormones due to 11β-oxidation of cortisol and corticosterone to their 11-oxo derivatives. Although the immunosuppressive and anti-inflammatory effects of glucocorticoids are well known, the relationship between inflammation and local metabolism of glucocorticoids is not well understood. In this study, we demonstrated that colitis induced by dextran sulfate sodium modulates colonic 11HSD1. Experimentally induced intestinal inflammation stimulated colonic $NADP^+$-dependent but not NAD^+-dependent 11HSD activity. Colonic 11HSD1 mRNA was increased, whereas 11HSD2 mRNA was not changed. Additional parallel studies revealed a similar pattern of 11HSD1 mRNA induction in mesenteric lymph nodes and intestinal intraepithelial lymphocytes, but not in spleen and peritoneal macrophages. These data suggest that inflammation modulates local metabolism of glucocorticoid and support the notion that pre-receptor regulation of endogenous corticosteroids might play a role in inflammatory processes.

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Introduction

Glucocorticoids are known to be essential modulators of immune and inflammatory processes. They influence the development and effector functions of the immune system, trafficking of immune cells through the vascular bed, and chemotaxis. At the cellular level, they modulate maturation, differentiation, proliferation, and activation of immune cells (Ashwell *et al.* 2000, Webster *et al.* 2002). One of the main effects of glucocorticoids is the downregulation of pro-inflammatory cytokines. These cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1), have been shown to link inflammation with glucocorticoid production by stimulating the hypothalamic–pituitary–adrenal axis and elevating thereby the plasma glucocorticoid concentration (Besedovsky & del Rey 1996). However, the biological activity of glucocorticoids depends not only on their plasma concentration, the number of receptors, and the responsiveness of the target cell, but also on the local metabolism of glucocorticoids catalyzed by 11β-hydroxysteroid dehydrogenase (11HSD), which can change the concentration of active glucocorticoids within tissues and/or target cells.

Two isoforms of 11HSD have been characterized. Isoform 2 (11HSD2) is a high-affinity NAD^+-dependent enzyme that operates exclusively as an oxidase inactivating biologically active glucocorticoids cortisol and corticosterone to their 11-oxo derivatives cortisone and 11-dehydrocorticosterone respectively (Stewart & Krozowski 1999). In contrast, 11HSD1 is a low-affinity NADP(H)-dependent oxido-reductase whose reductase activity has been found in various intact cells (Seckl & Walker 2001), but alterations in the $NADP^+$/NADPH redox potential governed by the metabolism of glucose-6-phosphate via hexose-6-phosphate dehydrogenase seem to determine whether 11HSD1 operates as a reductase or an oxidase (Hewitt *et al.* 2005). Thus, 11HSD2 decreases the local concentration of active gluco-corticoids, whereas 11HSD1 increases it due to regeneration of biologically active steroids from the circulating inactive 11-oxo metabolites or decreases it due to oxidation of active glucocorticoids (Stewart & Krozowski 1999, Seckl & Walker 2001, Hewitt *et al.* 2005). Exposure to pro-inflammatory stimuli such as TNF-α and IL-1β increases 11HSD1 expression and enzymatic activity in some cells, while inducing a decrease of 11HSD2 in others (Cai *et al.* 2001, Cooper *et al.* 2001, Heiniger *et al.* 2001, Thieringer *et al.* 2001, Tomlinson *et al.* 2001). The biological significance of this process was shown recently (Escher *et al.* 1997, Thieringer *et al.* 2001, Zhang *et al.* 2005).

With regard to the colon, 11HSD2 is expressed in epithelial cells, whereas 11HSD1 is localized in the cells of lamina propria (Whorwood *et al.* 1994). This matches with the findings of 11HSD1 in fibroblasts (Hammami & Siiteri

1991), macrophages (Thieringer *et al.* 2001), and lympho-cytes (Zhang *et al.* 2005). Consistent with the effect of TNF-α and IL-1β on 11HSD1 and 11HSD2, we have shown in a rat model of colitis that 11HSD1 mRNA expression and 11-reductase activity increased, whereas 11HSD2 mRNA expression and 11-oxidase activity decreased during intestinal inflammation (Bryndová *et al.* 2004). Considering that colitis is accompanied by activation of mucosal immune cells and increased recruitment of leucocytes from the vascular space (Elson *et al.* 1995), one can hypothesize that the link between the upregulation of colonic 11HSD1 mRNA and the increased ability of the tissue to reduce 11-dehydrocortico-sterone to corticosterone might be the cells of the intestinal immune system. To address this question, we used the dextran sulfate model of murine colitis and studied the changes of 11HSD1 in colon and immune cells during intestinal inflammation.

Materials and Methods

Animals and preparation of immune cells

Female 3-month-old mice Balb/c (Velaz, Prague, Czech Republic) were used in this study. Experimental colitis was induced by adding 3% (w/v) dextran sulfate sodium (DSS, MW 36 000–50 000; ICN Biomedicals Inc., Cleveland, Ohio, USA) in drinking water (Okayasu *et al.* 1990). Mice were treated with DSS for 7 days and subsequently killed. Control animals received only tap water. The mice were killed by decapitation and the colon, spleen, and mesenteric lymph nodes were excised. Macrophages were collected by a peritoneal lavage and intraepithelial lymphocytes (IEL) as originally described by Lefrancois (1992) with some modifications. Briefly, the inflamed part of colon was dissected and the lumen gently flushed with cold physiologi-cal saline (4 °C). The intestine was then incised longitudinally and cut into 5 mm pieces. The pieces were incubated in flasks containing RPMI-1640 medium supplemented with fetal bovine serum (12·5%) for 20 min at 37 °C with stirring. The incubation step was repeated thrice, the supernatants were combined, and IEL were separated from epithelial cells by

discontinuous Percoll gradient at the interface 67%/44%. The harvested cells were washed in RPMI medium and used for further analysis.

The animal study was approved by the Animal Care and Use Review Committee of the Czech Academy of Sciences.

Evaluation of colitis

The clinical assessment of DSS-treated animals included body weight, colon length, evaluation of stool consistency, and the presence of blood in the stool. A clinical disease activity index representing the sum of separate scores ranging from 0 to 4 was calculated using the following parameters: body weight decrease (0, less than 5% decrease; 1, 5–10%; 2, 10–20%; 4, more than 20%), stool consistency (solid 0, loose 2, diarrhea 3), and bleeding (none 0, macroscopic in colon 2, blood adhering to the anus 4) as described previously (Bendjelloul *et al.* 2000).

Quantitative analysis of 11HSD and cytokine RNA

Total RNA from the colon was extracted by the guanidinium thiocyanate method. The isolated RNA was treated with DNase (Promega) to remove potential contamination by genomic DNA as mentioned earlier (Mazancová *et al.* 2003). Total RNA from the spleen, mesenteric lymph nodes, macrophages, and IEL was obtained using GeneElute Mammalian Total RNA Miniprep Kit (Sigma). cDNA was synthesized from 5 μg RNA and M-MLV Reverse Tran-scriptase reagents (Invitrogen GmbH). Amplification of the target cDNA was performed in the LightCycler (Roche) as previously reported (Mazancová *et al.* 2005) using QuantiTect Sybr Green PCR Kit (Qiagen GmbH) and the primers given in Table 1. Results were analyzed with LightCycler software using the second derivative maximum method to set C_P. For the quantification of the target genes *11HSD1*, *11HSD2*, *TNF-α*, and *IL-1β*, we performed the quantitative compari-son of several candidate reference genes to select the most stable genes for gene normalization. The panel of five reference genes generally used in many physiological and pathophysiological conditions, such as β-actin (*ACTB*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*),

Table 1 Primers used for PCR

Gene	Sense (5′→3′)	Antisense (5′→3′)
11HSD1	GGTAGTGTCTCGCTGCCTTGAA	CACGTTGACCTTGGTTATGTAGAGTT
11HSD2	CCGGTTGTGACACTGGTTTTG	GGGGTATGGCATGTCTCCTG
TNF-α	GGACAAGGCTGCCCCGACTAC	TCTGAGCCATAATCCCCTTTC
IL-1β	TCCCAAGCAATACCCAAAGAAGAA	ATCAGAGGCAAGGAGGAAACACAG
β-actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG
Ppib	AGGGGAGATGGCACAGGAGGAAAGAGC	ACCCAGCCAGGCCCGRAGTGC
EF1a	TGACAGCAAAAACGACCCACCAAT	GGGCCATCTTCCAGCTTCTTACCA
RP13A	CTCCCGAGGCCCCTACCACTT	GGCCTTTTCCTTCCGTTTCTCCTC
HPRT	GCAGTCCCAGCGTCGTG	TAATGTAATCCAGCAGGTCAGC

ribosomal protein L13a (*RPL13A*), elongation factor 1α (*EF1A*), and peptidylpropyl isomerase B (*cyclophilin B; PPIB*), were tested (Table 1). Because of the high concentration of β–actin and 11HSD2, the samples were diluted 1/1000 before analyses of these RNA species. For other analyses, 1/10 pre–diluted cDNA was used as a template for PCR. Calibration curves were generated for each pair of primers from serial dilutions of standard cDNA. After statistical analysis of reference genes, the data of target gene expression were normalized according to the normalization factor calculated by the geNorm applet (Vandesompele *et al.* 2002).

The expression of 11HSD1 mRNA in IEL was determined by the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The probes and primers used for this experiment were developed as TaqMan Gene Expression Assays by Applied Biosystems. The reaction was carried out in a final volume of 20 μl using TaqMan Universal PCR Master Mix with AmpEraseUNG (Applied Biosystems) and Expression Assay. Target 11HSD1 mRNA was achieved using calibration curve method and the amount of mRNA was normalized to the level of 18S rRNA (Bas *et al.* 2004).

Enzyme activity assays

Colon homogenates were prepared in ice-cold buffers containing 10 mM Tris, 250 mM sucrose (pH 8·5; 11HSD2 assay) or 10 mM Tris, 5 mM EDTA, 0·5% Triton X-100 (pH 7·5; 11HSD1 assay). After centrifugation at 500 ***g*** for 15 min, the supernatant was obtained and protein concentration was measured using the Bradford technique (Bradford, 1976). 11HSD1 and 11HSD2 activities were measured as $NADP^+$- and NAD^+-dependent 11β-oxidation of corticosterone according to Livingstone & Walker (2003) and Gomez–Sanchez *et al.* (2003). 11HSD1 activity was measured in incubation buffer containing 50 mM Tris, 100 mM KCl, 1 mM $NADP^+$, 480 nM corticosterone, and 20 nM 1,2,6,7-[^{3}H]corticosterone (pH 7·5). 11HSD2 activity was determined in a similar way, with 50 mM Tris, 100 mM KCl, 1 mM NAD^+, and 20 nM 1,2,6,7-[^{3}H]corticosterone (pH 8·5). The amounts of protein and the incubation times were determined in preliminary experiments to establish the optimal conditions, in order to work in the linear portion of the enzyme reaction. Steroids were extracted from the incubation buffer by SepPak cartridges (Waters, Milford, MA, USA) and separated by HPLC with on-line detection using a flow-cell detector (Radiomatic 150TR, Canberra Packard, Meriden, CT, USA). The separation was performed in a C18 column using a water methanol gradient (for details see Pácha *et al.* 2004).

Data analysis

All data are expressed as means ± S.E.M. or medians with 25th–75th percentile values. The distribution–fitting procedure according to Shapiro–Wilk's *W*-test of normality was applied

and the comparison between the control animals and the mice with colitis was analyzed using unpaired Student's *t*-test or Mann–Whitney *U*-test. The values $P < 0·05$ were considered statistically significant. For stability comparison of candidate reference genes and the calculation of normalization factor, the geNorm program was applied after conversion of C_P values into relative quantities (Vandesompele *et al.* 2002). Using this approach, the normalization factor based on two candidate reference genes was calculated. Statistical analysis was performed using the statistical software Statistica v.6 (StatSoft Inc., Tulsa, OK, USA).

Results

The mice with colitis developed loose stools or diarrhea associated with blood in the stool and decreased colon length and body weight. Mortality was 10%. The index of disease activity is given in Table 2.

To investigate whether metabolism of glucocorticoids is modulated in inflamed tissue, we used enzyme assay and quantitative reverse transcription (RT)-PCR. First, we evaluated the expression levels of five putative reference genes in the colon of healthy mice and in animals with colitis. These genes displayed a relatively wide range of C_P (Fig. 1). Using the unpaired Student's *t*-test or Mann–Whitney *U*-test, significant differences in gene expression between healthy and inflamed colon were observed for EF1A and RPL13A. The geNorm program was then used to calculate the gene expression stability measure M of the remaining genes and the normalization factor based on the geometric average of the two reference genes (Vandesompele *et al.* 2002). We found ACTB and PPIB to be the most convenient reference genes and thus these two genes were used for normalization of mRNA expression levels.

To determine whether 11HSDs are modulated during inflammation, we measured colonic 11HSD1 and 11HSD2 mRNAs in control animals and in mice with colitis. As shown in Fig. 2, both groups of mice were found to express 11HSD1 and 11HSD2 transcripts and the inflammation affected the expression. Colitis upregulated 11HSD1 mRNA but did not

Table 2 Disease activity of DSS-induced colitis in colon

	Controls	Acute colitis
Symptom score	0·0 (0·0–0·0)	8·0 (6·0–10·0)*
Diarrhea score	0·0 (0·0–0·0)	3·0 (3·0–3·0)*
Blood score	0·0 (0·0–0·0)	4·0 (0·0–4·0)*
Body weight change	2·0 ± 0·8	−11·4 ± 1·5*
Colon length	9·9 ± 0·3	7·3 ± 0·1*

The data are given as medians and 25th–75th percentile values (score values) or mean ± S.E.M. (body weight change, colon length) based on records of 10 control and 9 DSS-treated animals. The score values were calculated as mentioned in Materials and Methods section, colon length is given in cm, and body weight change is given by dividing body weight at day 7 by body weight at day 0 (starting body weight) and is expressed as a percentage. *$P < 0·05$ compared with controls.
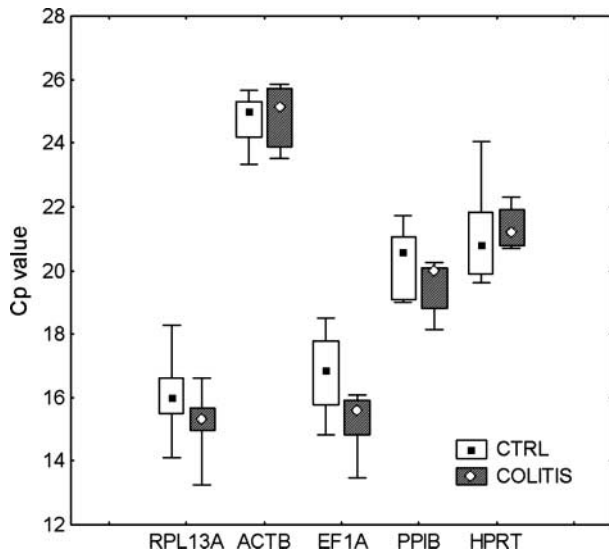
Figure 1 Expression levels of candidate reference genes in healthy and inflamed colon are shown as medians (points), 25th–75th percentiles (boxes), and ranges (whiskers). For the analyses, 1/1000 (ACTB) or 1/10 pre-diluted cDNA (RPL3A, EF1A, PPIB, and HPRT) was used as a template for PCR.
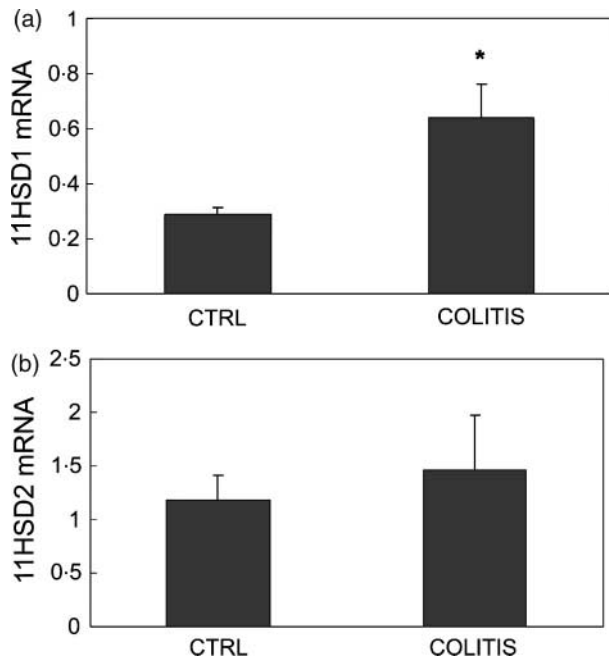

Figure 3 (a) NAD^+- and (b) $NADP^+$-dependent 11HSD activity in colonic homogenates of control mice and animals with colitis. Values are means±S.E.M. (control (CTRL), $n=10$; colitis, $n=10$). 11HSD activity is given in picomoles of corticosterone per hour per mg protein. *$P<0.05$ compared with controls.

significantly modulate the levels of 11HSD2 mRNA. To test whether the changes in transcript levels reflect changes in 11HSD1 and 11HSD2 enzyme activities, we used a colonic homogenate assay to analyze the NAD^+- and $NADP^+$-dependent conversion of corticosterone to 11-dehydrocorticosterone. The colon of controls and the mice with colitis had similar level of NAD^+-dependent 11HSD activity, but $NADP^+$-dependent 11HSD activity was significantly increased in inflamed tissue (Fig. 3). To verify the presence of colonic inflammation in this tissue, we analyzed gene expression of pro-inflammatory cytokines, TNF-α and IL-1β mRNAs. As shown in Table 3, the level of IL-1β transcript was significantly increased in inflamed colon but the level of TNF-α transcript was changed much less, in a similar way as in the study of Egger *et al*. (2000) and Kwon *et al*. (2005).

Next, we evaluated the changes in the level of 11HSD1 mRNA in the cells of gut-associated lymphatic tissue. There was a significant increase in 11HSD1 mRNA in IEL isolated

Figure 2 (a) Colonic 11HSD1 mRNA and (b) 11HSD2 mRNA levels in control mice and in animals with colitis. The relative expression levels were normalized against normalization factor from the two most stable reference genes provided by geNorm (for details see the text). The data are given as means±S.E.M. (control (CTRL), $n=10$; colitis, $n=9$). *$P<0.05$ compared with controls.
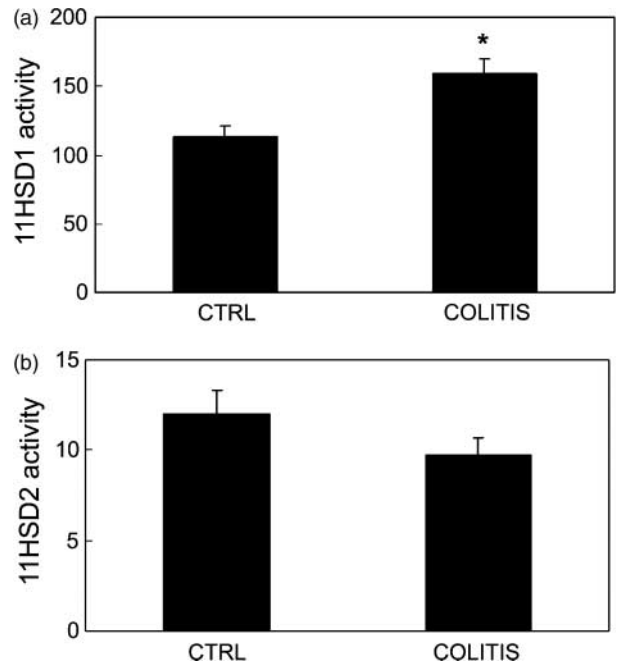
Table 3 Expression of TNF-α and IL-1β mRNAs in colon

	Controls	Acute colitis
TNF-α mRNA	0·202±0·063	0·258±0·61
IL-1β mRNA	0·023±0·002	0·356±0·183*

The data are given as means±S.E.M. of 10 control and 9 DSS-treated animals. The expression of cytokines is given in arbitrary units. *$P<0.05$ compared with controls.
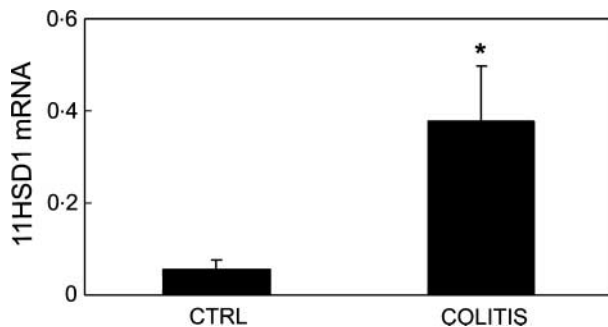
Figure 4 The relative expression levels of 11HSD1 mRNA in murine intestinal intraepithelial lymphocytes during DSS-induced colitis. Quantification of *11HSD1* gene expression was performed relative to the expression of 18S rRNA. $*P < 0.05$ compared with controls (control (CTRL), $n = 5$; colitis, $n = 9$).

from animals with colitis (Fig. 4). Consistent with upregulation of 11HSD1 mRNA in IEL, the lymphatic mesenteric nodes that contain large numbers of lymphocytes coming from the intestine demonstrated upregulation of 11HSD1 mRNA (Fig. 5), whereas 11HSD2 transcript was undetectable in the same nodes. In contrast to mesenteric lymph nodes, 11HSD1 transcript in spleen was not changed during colitis. Finally, to establish the potential contribution of macrophages to upregulation of colonic 11HSD1 during inflammation, the peritoneal macrophage 11HSD1 transcript was quantified. 11HSD1 mRNA was detectable in macrophages, but this transcript was not affected by colitis (controls: 0.56 ± 0.05 ($n = 10$); colitis: 0.48 ± 0.04 (9)). Taken together, these findings demonstrate similar changes in 11HSD1 mRNA in the colon, mesenteric lymphatic nodes, and IEL, but not in the spleen and peritoneal macrophages.

Discussion

The present study revealed that inflammation is associated with changes in 11HSD1 mRNA expression and enzyme activity in
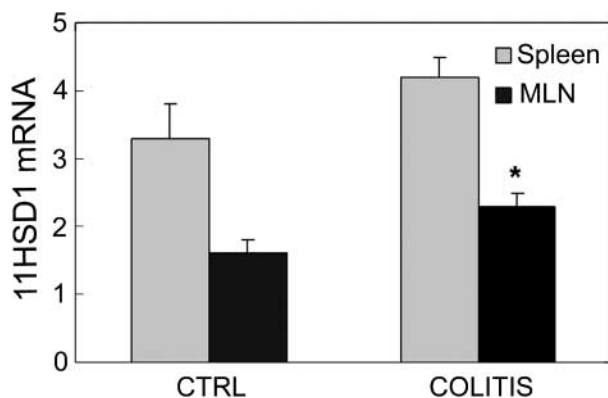

Figure 5 Expression of 11HSD1 in mesenteric lymph nodes (MLN) and spleen. The values were expressed as mentioned in Fig. 2. $*P < 0.05$ compared with controls (control, $n = 10$; colitis, $n = 9$).

colon. The inflamed colon upregulated 11HSD1 transcript and $NADP^+$-dependent 11HSD activity without any significant changes in 11HSD2 transcript and NAD^+-dependent 11HSD activity. As 11HSD2 is expressed only in epithelium, whereas 11HSD1 is expressed in the submucosal layer (Whorwood *et al.* 1994), it is likely that changes in 11HSD1 proceed in the intestinal compartment of lamina propria or gut-associated lymphatic tissue and not in the epithelium. 11HSD1 was found in macrophages (Thieringer *et al.* 2001), T cells (Zhang *et al.* 2005), and fibroblasts (Hammami & Siiteri 1991), and we have found an increased level of 11HSD1 mRNA in IEL and mesenteric lymphatic nodes of mice with colitis. The question is what is the direction of 11HSD1 reaction in intact cells *in vivo*?. This direction (11β-reduction or 11β-oxidation) depends on the ratio of NADPH/$NADP^+$, which is determined by hexose-6-phosphate dehydrogenase and pentose-phosphate pathway (Atanasov *et al.* 2004, McCormick *et al.* 2006). Thus, it is difficult to anticipate the reaction direction of 11HSD1 in inflamed colon. Considering that 11HSD1 operates as a reductase in activated macrophages (Gilmour *et al.* 2006), lymphocytes (Zhang *et al.* 2005), and dendritic cells (Freeman *et al.* 2005), it is likely that colonic 11HSD1 is increased by a shift in favor of the reductase activity. Further studies are needed to clarify the role of 11HSD isoforms of colonic wall *in vivo* by cellular distribution of 11HSDs in cell types of colonic mucosa and submucosa during inflammation and the effect of NADPH depletion during oxidative stress on the direction of the reaction catalyzed by 11HSD1 in immune cells.

The exact mechanism of inflammation induced by DSS is not yet fully elucidated, but the pathogenesis seems to depend on the interaction between local immune reaction and environmental factors because animal models of inflammatory bowel disease reared in germfree conditions did not develop the disease (Tlaskalová-Hogenová 1997, Hudcovic *et al.* 2001, Elson & Cong 2002). Drinking of DSS generates in murine colon the upregulation of pro-inflammatory cytokines as well as reactive oxygen and nitrogen species and infiltration by polymorphonuclear and mononuclear cells (Okayasu *et al.* 1990, Kojouharoff *et al.* 1997, Arai *et al.* 1998). Previous data and our findings suggest that these processes are accompanied by upregulation of 11HSD1 in the cells of gut-associated lymphatic tissue. First, activated macrophages and lymphocytes acquire increased capacity of glucocorticoid reactivation via 11HSD1 (Zhang *et al.* 2005, Gilmour *et al.* 2006). Secondly, our data show that $NADP^+$-dependent but not NAD^+-dependent activity is increased in inflamed colon. Thirdly, the level of 11HSD1 transcript is increased not only in inflamed colon but also in IEL and in mesenteric lymphatic nodes. This upregulation of 11HSD1 is presumably induced by the pro-inflammatory cytokines, whose levels of transcript and protein are increased in colon of DSS-treated mice (Arai *et al.* 1998, Egger *et al.* 2000, Obermeier *et al.* 2002, Kwon *et al.* 2005). The cytokines TNF-α and IL-1β are known to increase 11HSD1 mRNA and 11-reductase activity in various cell types, such as glomerular mesangial cells (Escher *et al.*

1997), osteoblasts (Cooper *et al.* 2001), adipocytes (Tomlinson *et al.* 2001), and aortic smooth muscle cells (Cai *et al.* 2001). In addition, pro-inflammatory cytokines are important inducers of nitric oxide generation in macrophages and intestinal epithelial cells, and the interaction between the NO system and the 11HSDs has recently been demonstrated (Kolios *et al.* 1996, Saito & Nakano 1996, Sun *et al.* 1997, Ruschitzka *et al.* 2001).

It is well recognized that glucocorticoids effectively modulate a number of immunological processes, including positive or negative regulation of cytokine production (Hennebold *et al.* 1996, Ashwell *et al.* 2000). Therefore, changes in the metabolism of glucocorticoids within peripheral lymphatic organs and in immune cells could modulate not only the suppression of cell activation by pro-inflammatory cytokines but also other immunomodulatory processes (Elenkov & Chrousos 1999, McKay & Cidlowski 1999). Using glomerular mesangial cells exposed to IL-1β and TNF-α, it was demonstrated that the release of phospholipase A2, a key enzyme producing inflammatory mediators, is decreased by 11HSD1 activity (Escher *et al.* 1997). Similarly, the pharmacological inhibition of 11HSD *in vivo* greatly enhanced the susceptibility to progressive bacterial diseases and these changes in resistance following 11HSD inhibition correlated with changes in the patterns of inducible cytokines in lymphocytes and macrophages (Hennebold *et al.* 1997). Given the central role of 11HSD1 in glucocorticoid action, we can speculate that the increased expression of this enzyme may serve to enhance the exposure of immune cells to active glucocorticoids via the paracrine and/or intracrine pathway.

In summary, our observations are consistent with the notion that inflammation is associated with changes in 11HSD1. Although the mechanism and accurate function of 11HSD1 upregulation is equivocal, the findings suggest that the bioavailability of glucocorticoids in inflamed colon differs from the healthy tissue.

Acknowledgements

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