The nuclear receptor LRH-1 critically regulates extra-adrenal glucocorticoid synthesis in the intestine

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The Journal of Experimental Medicine

The nuclear receptor liver receptor homologue-1 (LRH-1, NR5A2) is a crucial transcriptional regulator of many metabolic pathways. In addition, LRH-1 is expressed in intestinal crypt cells where it regulates the epithelial cell renewal and contributes to tumorigenesis through the induction of cell cycle proteins. We have recently identified the intestinal epithelium as an important extra-adrenal source of immunoregulatory glucocorticoids. We show here that LRH-1 promotes the expression of the steroidogenic enzymes and the synthesis of corticosterone in murine intestinal epithelial cells in vitro. Interestingly, LRH-1 is also essential for intestinal glucocorticoid synthesis in vivo, as LRH-1 haplo-insufficiency strongly reduces the intestinal expression of steroidogenic enzymes and glucocorticoid synthesis upon immunological stress. These results demonstrate for the first time a novel role for LRH-1 in the regulation of intestinal glucocorticoid synthesis and propose LRH-1 as an important regulator of intestinal tissue integrity and immune homeostasis.

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Liver receptor homologue-1 (LRH-1, NR5A2) is a nuclear receptor with sequence homology to steroidogenic factor-1 (SF-1, NR5A1) (for review see reference 1). In addition to their homology, SF-1 and LRH-1 bind to identical DNA consensus sequences and are able to bind phospholipids (1–5). Apart from these shared properties, LRH-1 and SF-1 have a quite differential tissue expression pattern and thus likely different functions. SF-1 expression is confined to steroidogenic tissues and the hypothalamo–pituitary–adrenal axis, where it regulates development, differentiation, steroidogenesis, and sexual determination (1). Although SF-1 is abundantly expressed in the adrenals, it is absent in the intestinal mucosa. In contrast, LRH-1 is expressed in intestine, liver, exocrine pancreas, and the ovary where it plays an important role in development, reverse cholesterol transport, bile acid homeostasis, and steroidogenesis (1). In the intestinal mucosa, LRH-1 is predominantly expressed by crypt cells, where it regulates the expression of cyclin D1 and E1 and thereby promotes epithelial cell proliferation and crypt cell renewal (6). Consequently, LRH-1 has also been implicated in the development of colon carcinomas (7).

Glucocorticoids are steroids with important immunoregulatory functions (8). Endogenous glucocorticoid synthesis is substantially regulated by the transcriptional control of steroidogenic enzymes of the cytochrome P450 gene family (CYP genes). Previous studies have shown that the nuclear receptors SF-1 and LRH-1 are potent regulators of some of these genes in the adrenals and ovaries (1, 9–12). Although the adrenals are the most important source of glucocorticoids, there is increasing evidence for extra-adrenal glucocorticoid synthesis in other organs and tissues (13–15). We have recently identified the intestinal epithelium as a potent source of extra-adrenal glucocorticoid synthesis (16). Intestinal glucocorticoid production is induced upon immunological stress through the induction of steroidogenic enzymes. Importantly, intestinal glucocorticoids

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The online version of this article contains supplemental material.

mediate an important regulatory feed-back loop and critically regulate intestinal immune responses. In the absence of intestinal glucocorticoids, antigen-specific T cells become overactivated during viral infections, indicating an immunosuppressive role of locally produced glucocorticoids (16).

The factors that regulate intestinal glucocorticoid synthesis are unknown thus far. As LRH-1 and steroidogenic enzyme expression is confined to the crypt cells of the intestinal mucosa (6, 16), we investigated the role of LRH-1 in the regulation of intestinal glucocorticoid synthesis. We show here that LRH-1 expression is induced in the intestine upon immune stimulation and parallels the induction of the genes of the steroidogenic enzymes CYP11A1 and CYP11B1. Overexpression of LRH-1 in murine intestinal epithelial cells strongly induces the transcriptional activation of these genes and promotes the synthesis of corticosterone. Importantly, LRH-1 is critical for intestinal glucocorticoid synthesis in vivo, as LRH-1 halplo-insufficiency abrogates immune cell–induced expression of CYP11A1 and CYP11B1 and associated corticosterone synthesis in the intestinal mucosa. This report demonstrates for the first time that LRH-1 is involved in glucocorticoid synthesis. As SF-1 expression is absent in the intestinal epithelium, we propose that LRH-1 has a unique role in the regulation of extra-adrenal glucocorticoid synthesis and immune regulation in the intestinal mucosa.

RESULTS AND DISCUSSION

LRH-1 expression is induced upon T cell activation in the intestinal mucosa

LRH-1, but not SF-1, has been previously reported to be abundantly expressed in intestinal crypt cells (6). As the same cells also express steroidogenic enzymes and produce the bioactive glucocorticoid corticosterone (16), we aimed at investigating the role of LRH-1 in intestinal glucocorticoid synthesis. Intestinal steroidogenic enzyme expression and glucocorticoid synthesis is induced upon injection of an agonistic T cell–activating anti-CD3 antibody (16). We thus analyzed the expression of LRH-1 in the small intestinal tissue from control and anti-CD3–treated mice, and compared it to that in adrenal glands. As described previously, LRH-1 expression levels in the adrenal glands were found to be very low (1). In marked contrast, even basal levels of LRH-1 in the intestine were >200 times higher than those found in adrenals. Interestingly, injection of anti-CD3 resulted in an additional threefold induction of intestinal LRH-1 levels (Fig. 1). Similarly, infection of mice with the lymphocytic choriomeningitis virus caused an up-regulation of intestinal LRH-1 and CYP11B1 expression (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060357/DC1).

LRH-1 promotes expression of steroidogenic enzymes and glucocorticoid synthesis

As anti-CD3–mediated increase of intestinal LRH-1 expression correlated with the induction of intestinal steroidogenic enzyme expression and glucocorticoid synthesis (16), we assessed whether LRH-1 overexpression can promote the

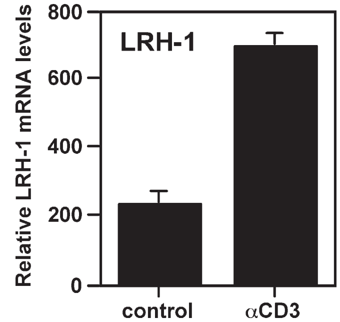

Figure 1. LRH-1 expression in intestinal mucosa. Wild-type mice were injected with PBS (control) or anti-CD3 antibody (αCD3) for 3 h. Small intestinal tissue was isolated and LRH-1 expression was measured by quantitative real-time RT-PCR. LRH-1 expression levels in adrenal glands isolated from control mice were used for normalization. Mean values of LRH-1 expression levels ± SD normalized to adrenals of three mice per group are shown.

expression and activity of steroidogenic enzymes in the murine intestinal epithelial cell line mICcl2 that displays crypt-like features (17). We thus transfected these cells with the CYP11A1 or CYP11B1 luciferase reporter constructs in the absence or presence of increasing amounts of LRH-1 expression plasmid. The CYP11A1 gene encodes for P450 ssc, the rate-limiting enzyme in the glucocorticoid synthesis converting cholesterol to pregnenolone, whereas the CYP11B1 gene encodes for 11β-hydroxylase, the enzyme catalyzing inactive 11-deoxycorticosterone to active corticosterone. Ectopic expression of LRH-1 dose-dependently induced CYP11A1 promoter activity and resulted in a sixfold increase of basal activity. The CYP11B1 promoter was found to be even more responsive to induction by LRH-1 and an up to 25-fold induction could be detected (Fig. 2, A and B). These data were confirmed by analysis of endogenous CYP11A1 and CYP11B1 mRNA expression. Transfection of mICcl2 cells with LRH-1 resulted in a strong increase of endogenous levels of both gene transcripts (Fig. 2, C and D).

Most intriguingly, the increase in expression of these key enzymes in the glucocorticoid synthesis pathway observed after LRH-1 transfection of mICcl2 cells was also accompanied with a robust release of corticosterone (Fig. 2 E). These data demonstrate that increased expression of LRH-1 is sufficient to promote the expression of steroidogenic enzymes and thereby trigger the glucocorticoid synthesis in intestinal epithelial cells.

Mutation of the LRH-1 response element inhibits LRH-1–induced CYP11A1 and CYP11B1 promoter activity

This induction of CYP11A1 and CYP11B1 transcription is likely the effect of direct binding to the promoter of these genes as putative LRH-1/SF-1 consensus sequences have been identified in both the CYP11A1 and CYP11B1 promoters (9, 11, 18, 19). To confirm the importance of these response elements in the LRH-1–mediated induction of CYP11A1 and CYP11B1 promoter activity, we mutated the predicted proximal response

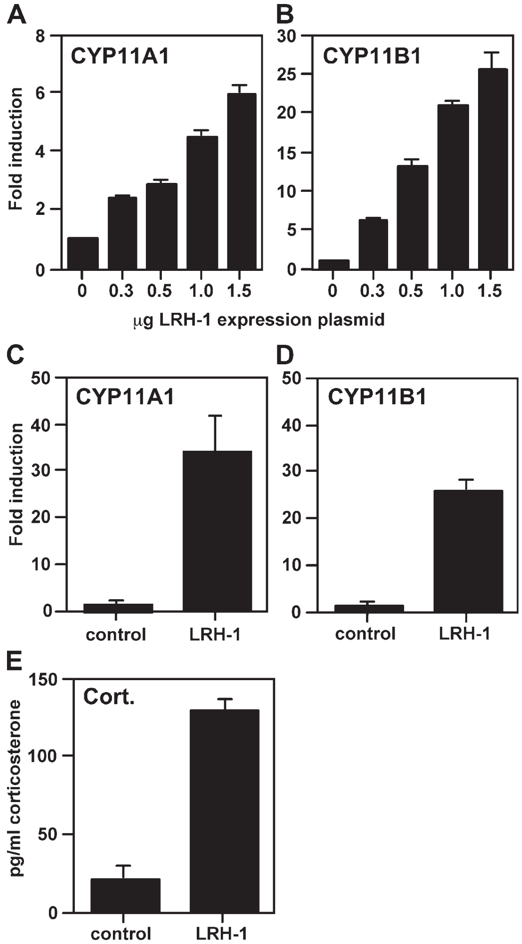

Figure 2. LRH-1 induces steroidogenic enzyme expression and glucocorticoid synthesis. (A and B) The murine intestinal epithelial cell line mICcl2 was transfected with the murine CYP11A1 (A) or CYP11B1 (B) promoter reporter construct and increasing amounts of LRH-1 expression plasmid. Luciferase activity was measured and normalized to CYP11A1 and CYP11B1 reporter construct alone, respectively. Mean values of triplicates ± SD of a typical experiment out of three are shown. (C and D) mICcl2 cells were transfected with pCMX (control) or pCMX-LRH-1 (LRH-1) (1 μg) and endogenous CYP11A1 (C) and CYP11B1 mRNA expression was measured by real-time RT-PCR. Mean values of triplicates ± SD of a typical experiment out of three are shown. (E) mICcl2 cells were transfected as described before and corticosterone was measured in the supernatant after 16 h. Mean values of triplicates ± SD of a typical experiment out of three are shown.

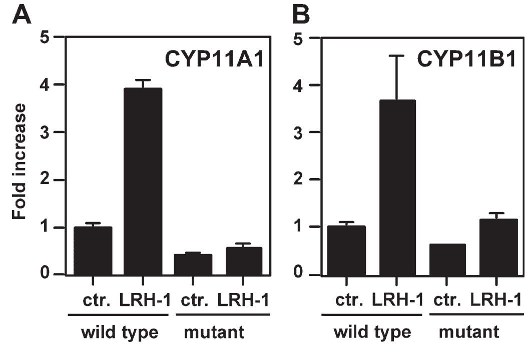

Figure 3. Mutation of LRH-1 response element inhibits LRH-1–induced CYP11A1 and CYP11B1 promoter activity. mICcl2 cells were cotransfected with wild-type or mutant CYP11A1 (A) or CYP11B1 (B) reporter constructs (1 μg each), respectively, and control plasmid or LRH-1 expression plasmid (1 μg each). Luciferase activity was measured and normalized to wild-type CYP11A1 and CYP11B1 reporter construct alone, respectively. Mean values of triplicates ± SD of a typical experiment out of two are shown.

element in the two promoter reporter constructs. In agreement with the notion that LRH-1 directly acts on these promoters via binding to these response elements, we observed an almost complete inhibition of LRH-1–induced CYP11A1 and CYP11B1 promoter activity (Fig. 3).

Endogenous LRH-1 promotes basal CYP11A1 and CYP11B1 promoter activity

As LRH-1 is abundantly expressed in normal intestinal epithelial cells and overexpression of LRH-1 strongly promotes corticosterone synthesis in mICcl2 cells, we next investigated whether endogenous expression of LRH-1 may be responsible for induction of basal CYP11A1 and CYP11B1 promoter activities in mICcl2 cells. Cells were thus transfected with CYP11A1 and CYP11B1 promoter reporter constructs and a dominant negative LRH-1 expression vector (DN LRH-1) comprising the DNA binding domain, but lacking the ligand-dependent transactivation domain. Competition of DNA binding of DN LRH-1 with endogenous LRH-1 dramatically inhibited basal CYP11A1 promoter activity and still substantially down-regulated basal CYP11B1 promoter activity (Fig. 4). These data suggest that endogenous LRH-1 expression levels contribute to basal expression levels of steroidogenic enzymes in intestinal epithelial cells.

Critical role of LRH-1 in intestinal glucocorticoid synthesis in vivo

We next investigated the role of endogenous LRH-1 in the induction of intestinal glucocorticoid synthesis in vivo using the LRH-1 halplo-insufficient (LRH-1$^{+/-}$) mouse model (6). LRH-1 is critical for embryonic development; homozygous LRH-1–deficient mice die in utero (6, 20, 21), whereas LRH-1$^{+/-}$ mice are viable and develop normally, allowing the investigation of LRH-1 in vivo. To study the role of LRH-1 in the regulation of intestinal glucocorticoid synthesis in vivo, we used the previously established model of anti-CD3–induced steroid synthesis (16). LRH-1$^{+/+}$ and LRH-1$^{+/-}$ mice were treated with PBS control or anti-CD3 antibody leading to strong T cell activation and subsequent intestinal glucocorticoid synthesis. As predicted, injection of anti-CD3 antibody substantially induced intestinal expression levels of LRH-1 in the small and large bowel of wild-type animals, but not of LRH-1 haplo-insufficient animals (Fig. 5). In agreement with the expected role of LRH-1 in the regulation of steroidogenic enzymes, we also observed a strong anti-CD3–mediated induction of CYP11A1

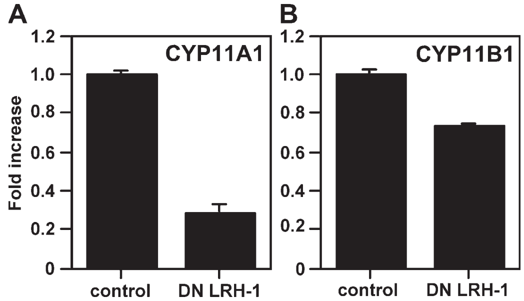

Figure 4. Endogenous LRH-1 promotes basal CYP11A1 and CYP11B1 promoter activity. mICcl2 cells were cotransfected with CYP11A1 (A) or CYP11B1 (B) reporter constructs, and control plasmid or dominant negative LRH-1 (DN LRH-1) expression plasmid. The inhibition of basal promoter activity of triplicates ± SD of a typical experiment is shown ($n = 3$).

and CYP11B1 expression in tissue from small and large intestine of wild-type animals, which was substantially reduced in the intestinal tissue isolated from LRH-1$^{+/-}$ animals (Fig. 5). Finally, ex vivo–cultured small intestinal tissue from anti-CD3–treated LRH-1$^{+/+}$ mice resulted in a strong release of metyrapone-inhibitable corticosterone, as measured by radioimmunoassay (RIA). In marked contrast, no anti-CD3–driven increase in glucocorticoid synthesis was observed in the intestinal mucosa of LRH-1$^{+/-}$ mice (Fig. 5).

These findings show that LRH-1 is a central transcription factor for the expression of steroidogenic enzymes and the synthesis of corticosterone in the murine intestinal tissue. Importantly, although LRH-1 has already previously been implicated in the regulation of sex steroid synthesis pathways (e.g., by regulating the expression of CYP11A1 in granulosa cells of the ovary) (22), the present study represents to our knowledge the first report that demonstrates the involvement of LRH-1 in the synthesis of glucocorticoids. In the adrenal glands, regulation of glucocorticoid synthesis appears to be critically dependent on SF-1 (23). SF-1 deficiency results in the absence of adrenal glands and consequently in the absence of systemic glucocorticoids in the adult animal (for review see reference 24). As LRH-1 is only expressed at minimal levels in the adrenal glands, it is likely that LRH-1 cannot compensate for the lack of SF-1 in this organ. Accordingly, LRH-1$^{+/-}$ mice have normal serum glucocorticoid levels. Although SF-1–deficient mice lack adrenals and gonads, they have normal CYP11A1 expression in the placenta, indicating that LRH-1 may regulate placental CYP11A1 expression (12). Quite surprising is the observation that, although adult SF-1–deficient animals lack serum glucocorticoids, they have normal serum levels at the embryonic stage, demonstrating that other extra-adrenal sources contribute to the synthesis of systemic glucocorticoids (12). Based on our findings that LRH-1 critically regulates intestinal glucocorticoid synthesis, it is tempting to speculate that the embryonic intestinal mucosa may also secrete glucocorticoids into the blood stream in an LRH-1–dependent manner. In agreement with this notion, it was reported that CYP11A1 is expressed in the embryonic gut in the absence of SF-1 (25). Clearly, in the adult animal, SF-1 expression is absent in the intestinal epithelium and LRH-1 appears to represent the main regulator of intestinal glucocorticoid synthesis.

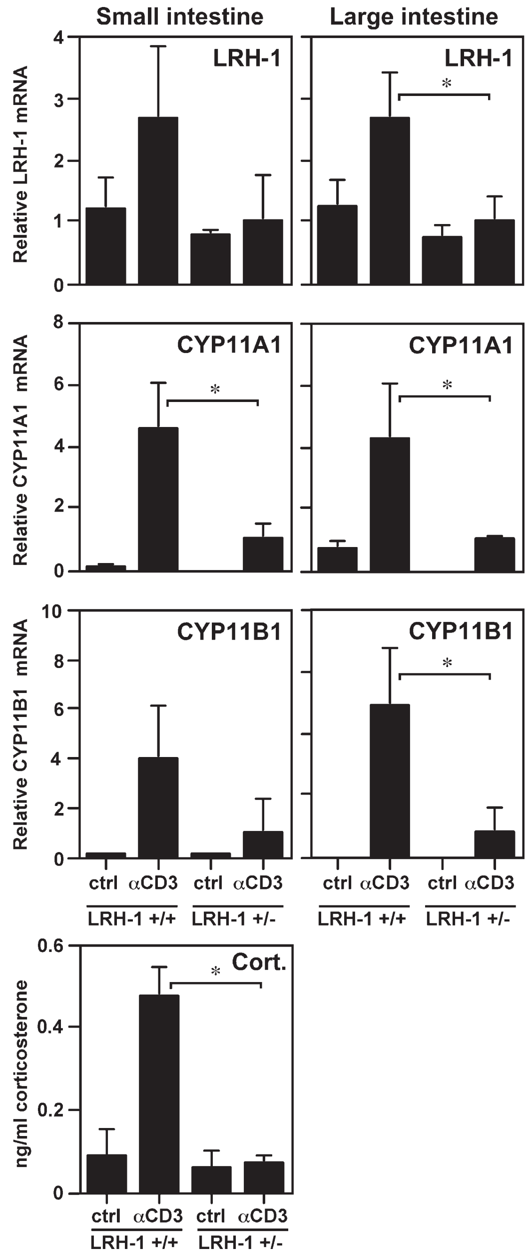

Figure 5. Endogenous LRH-1 expression is required for intestinal expression of steroidogenic enzymes and glucocorticoid synthesis in vivo. Wild-type LRH-1$^{+/+}$ mice and heterozygous LRH-1$^{+/-}$ mice were injected with PBS (ctrl) or anti-CD3 antibody (αCD3) for 3 h. Small and large intestinal tissues were isolated and LRH-1, CYP11A1, and CYP11B1 expression was measured by real-time RT-PCR and normalized to GAPDH. In addition, small intestinal tissue from control or anti-CD3–treated mice was cultured ex vivo for 6 h and corticosterone (Cort.) in the cell-free supernatant was measured by RIA as described in Materials and methods. Mean values ± SD of three mice per group of a typical experiment out of two are shown. *, $P < 0.05$.

LRH-1 likely regulates intestinal glucocorticoid synthesis not only at the level of CYP11A1 and CYP11B1 transcription. As LRH-1 and SF-1 have an overlapping target pattern, it is expected that also the expression of other steroidogenic enzymes may be regulated, directly or indirectly, by LRH-1. In addition, as SF-1 controls the availability of cholesterol by inducing the expression of steroidogenic acute regulatory protein (StAR), LRH-1 may similarly regulate steroid synthesis at the substrate level (cholesterol). A recent finding by Kim et al. supports this idea by demonstrating a role for LRH-1 in StAR expression in granulosa cells (22).

LRH-1 not only plays a critical role in the regulation of extra-adrenal intestinal glucocorticoid synthesis but also promotes intestinal epithelial cell renewal (i.e., by inducing the expression of the cell cycle proteins cyclin D1 and E1) (6). Although epithelial cell renewal and glucocorticoid synthesis have little in common at a first glance, there may be a link between these two LRH-1–regulated processes. Strong T cell activation and associated immunological responses in the intestinal mucosa lead to the release of proinflammatory cytokines, such as IFNγ and TNFα, which impair the intestinal epithelial barrier function (26). Similarly, T cell–mediated cytotoxicity can cause epithelial cell apoptosis (27) and thereby also leads to increased permeability of the epithelial layer. The resulting leakiness of the epithelial layer usually results in an increased stimulation of immune cells by luminal bacterial products, which further accelerates destructive inflammatory responses in the gut. Induction and activation of LRH-1 may not only reduce the epithelial layer leakiness and stimulation of immune cells by increasing epithelial cell renewal but may also control immune cell activation by the regulating intestinal glucocorticoid synthesis. We therefore suggest that LRH-1 plays an important role in the regulation of intestinal immune homeostasis.

MATERIALS AND METHODS

Cells and reagents. The murine intestinal epithelial cell line mICcl2 has been described previously (17). The culture medium consisted of Dulbecco's modified Eagle medium/Ham's F-12 12g/L (1:1, vol/vol, GIBCO BRL), NaHCO3 2.438g/L, 2% steroid-free FCS, 60 nmol/L sodium selenate, 5 μg/ml apo-transferrin, 10 ng/ml murine EGF, 1 nmol/L triiodothyronine, 5 μg/ml insulin, 2 mmol/L L-alanyl-L-glutamine, 20 mmol/L Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. The glucocorticoid synthesis inhibitor metyrapone was obtained from Sigma-Aldrich.

Plasmids. The expression construct for wild-type murine LRH-1 (pCMX-LRH-1) has been described previously (28). For the cloning of the dominant negative expression vector for murine LRH-1 (DN-LRH-1), cDNA sequences corresponding to aa 1–282 of mouse LRH-1 were PCR-amplified with primers 5′-GGAATTCTTCGCTAAGAATGTCTGCTAGT-3′ and 5′-GGAATTCTCAGTGACCATAGGGTTGGTAACCA-3′ from pCMX-LRH-1 and the amplification product was subsequently cloned into the EcoRI site of pCMX. For the promoter reporter constructs, 1.7 kb of the 5′-flanking region of the murine CYP11A1 (accession no. 13070) and CYP11B1 (accession no. NT 082172.1) genes were amplified by PCR from C57BL/6 genomic DNA using specific primers (CYP11A1 fw 5′-GCAAGGATCCTTCCTTCTCACAATCCTAAG-3′, rev 5′-GATCGTCGACAGTCTTTTAGCCAGCATAC-3′; CYP11B1 fw 5′-CTAAGGATCCGTGATTCTCTGATGGTCTCT-3′, rev 5′-CCTTGTCGACTTGTCATCTTCCTCTCTTCC-3′) and cloned into the HsLuc luciferase reporter construct (29) using BamHI and SalI restriction sites.

The predicted LRH-1/SF-1 core binding sites (AGGTCA) (19) at positions −138 and −221 of the CYP11A1 and the CYP11B1 promoter, respectively, were mutated (to taaTCA) by site-directed mutagenesis using a mutagenesis kit (Quick Change; Stratagene) and the following primers: mut mCYP11A1 fw 5′-GGGtaaTCACCG-3′, mut mCYP11A1 rev 5′-CGGTGAttaCCC-3′; mut mCYP11B1 fw 5′-ACCTGAaatCAG-3′, mut mCYP11B1 rev 5′-CTGattTCAGGT-3′.

CYP11A1 and CYP11B1 promoter assay. CYP11A1 or CYP11B1 wild-type and mutated reporter constructs, and β-galactosidase expression vector for transfection control, were cotransfected into mICcl2 cells using the calcium phosphate precipitation method. In some experiments, cells were cotransfected with different amounts of a wild-type or dominant negative murine LRH-1 expression vector. After overnight transfection, cells were washed and cultured for 16 h before lysis of the cells. β-galactosidase and luciferase activity assays were performed as described previously (30).

Induction and measurement of intestinal glucocorticoid synthesis. Intestinal glucocorticoid synthesis was induced as previously described (16). In brief, age- and sex-matched wild-type $LRH^{+/+}$ mice or heterozygous $LRH\text{-}1^{+/-}$ mice in the C57BL/6 background (6, 7) were injected i.p. with either PBS or 50 μg of anti–mouse CD3 antibody i.p. After 4 h, mice were killed and small intestinal tissue was isolated and cultured in the presence or absence of the glucocorticoid synthesis inhibitor metyrapone (200 μg/ml) for 6 h. After that, cell-free supernatant was harvested and corticosterone was measured by RIA. Results were expressed as the difference between samples cultured without metyrapone and samples cultured with metyrapone (metyrapone-inhibitable corticosterone synthesis) to correct for variable contamination with serum glucocorticoids. All animal experiments have been reviewed and approved by the review board of the State of Bern.

In some experiments, control or LRH-1–transfected mICcl2 cells were cultured for 16 h and corticosterone in cell-free supernatant was measured by RIA.

Detection of CYP11A1, CYP11B1, and LRH-1 mRNA by real-time RT-PCR. Small and large intestinal tissue from PBS or anti-CD3–injected $LRH\text{-}1^{+/+}$ or $LRH\text{-}1^{+/-}$ mice, or control or LRH-1–transfected mICcl2 cells were lysed in TRI reagent (Sigma-Aldrich) and RNA was isolated. RNA was DNase treated and 2 μg of RNA were reverse transcribed using a Taqman Gold RT kit obtained from Applied Biosystems. Real-time PCR was performed in an Applied Biosystems Real-time PCR 7500 machine using SYBR green and the following primers: mCYP11A1 forward 5′-CCAGCCCAACATTACCGAGAT-3′, reverse 5′-GACTTCAGCCCGCAGCAT-3′; mCyp11B1 forward 5′-CAATAGAAGCTAGCCACTTTGT-3′, reverse 5′-AGGGTGTGGAGGAACTTCAG-3′. For mLRH-1 and the house-keeping gene GAPDH amplification Quantitec primer assays obtained from QIAGEN were used. GAPDH was used to normalize CYP11A1, CYP11B1, and LRH-1 expression levels.

Statistical analysis. In some experiments, differences between groups were analyzed by unpaired Student's *t* test. Values of $P < 0.05$ were considered significant.

Online supplemental material. Fig. S1, describing viral infection-induced LRH-1 and CYP11B1 expression, is available at http://www.jem.org/cgi/content/full/jem.20060357/DC1.

The authors would like to thank the members of the Brunner lab and M. Matter for technical help and advice, J. Auwerx for the LRH-1-deficient mice and support, and J.-M. Zingg for help with the luciferase measurements.

This work was supported by research grants from the Swiss National Science Foundation (no. 31-65021.01 and 310000-110030) and the Crohn's and Colitis Foundation of America (1441) (to T. Brunner), and INSERM, CNRS, Hopitaux Universitaires de Strasbourg, ARC, and ACI-MRT (to K. Schoonjans).

The authors have no conflicting interests.

Submitted: 14 February 2006
Accepted: 31 July 2006

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