

Liver X receptors α and β regulate renin expression in vivo

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The renin-angiotensin-aldosterone system controls blood pressure and salt-volume homeostasis. Renin, which is the first enzymatic step of the cascade, is critically regulated at the transcriptional level. In the present study, we investigated the role of liver X receptor α (LXR α) and LXR β in the regulation of renin. In vitro, both LXRs could bind to a noncanonical responsive element in the *renin* promoter and regulated *renin* transcription. While LXR α functioned as a cAMP-activated factor, LXR β was inversely affected by cAMP. In vivo, LXRs colocalized in juxtaglomerular cells, in which LXR α was specifically enriched, and interacted with the *renin* promoter. In mouse models, renin-angiotensin activation was associated with increased binding of LXR α to the responsive element. Moreover, acute administration of LXR agonists was followed by upregulation of *renin* transcription. In LXR α ^{-/-} mice, the elevation of renin triggered by adrenergic stimulation was abolished. Untreated LXR β ^{-/-} mice exhibited reduced kidney *renin* mRNA levels compared with controls. LXR α ^{-/-}LXR β ^{-/-} mice showed a combined phenotype of lower basal renin and blunted adrenergic response. In conclusion, we show herein that LXR α and LXR β regulate renin expression in vivo by directly interacting with the *renin* promoter and that the cAMP/LXR α signaling pathway is required for the adrenergic control of the renin-angiotensin system.

Introduction

Renin is an aspartyl protease that catalyzes the cleavage of angiotensinogen to angiotensin I, the first and rate-limiting step of the renin-angiotensin cascade. Circulating renin is expressed in and released from specialized juxtaglomerular (JG) cells strategically located in the afferent arterioles of kidney glomeruli. By regulating the rate of angiotensin generation, renin plays a pivotal physiological role in salt-volume homeostasis and in the control of blood pressure. Increased renin expression and activity is associated with cardiovascular diseases such as hypertension, congestive heart failure, stroke, and kidney disease. Given its key physiological function, renin is highly regulated from transcription to secretion. The transcriptional regulation is particularly critical and complex, functioning through positive and negative regulatory elements located in the promoter and enhancer regions (1). Several transcription factors are capable of binding to these sites and can influence the expression of renin in vitro. However, the in vivo significance of these factors with respect to the physiology and pathophysiology of the renin-angiotensin-aldosterone system (RAAS) is unknown, since most of the current evidence is based on in vitro studies performed on cell lines with limited similarity to kidney JG cells.

Using immortalized cell lines, we have previously identified a member of the nuclear hormone receptor family, liver X receptor α

(LXR α , also known as NR1H3), as a potential regulator of renin expression. In vitro, LXR α can control the transcription of a cluster of genes including *renin* in a cAMP-dependent fashion (2, 3) by binding to a novel response element termed CNRE (an overlapping of a cAMP and a negative response element). In the *renin* promoter, this element is located in the proximal region (\approx 600 bp) and is conserved in the human and rodent genomes (4). Importantly, the CNRE element is distinct from the classical LXR response element termed DR4, which mediates all the currently described molecular and pathophysiological functions of LXRs (5). However, the actual relevance of these observations to kidney JG cells and the RAAS in vivo has been unclear so far. Also, the ability of the highly homologous and ubiquitously expressed LXR β (NR1H2) to bind CNRE and functionally regulate renin has not been examined previously. In this study, we used a comprehensive approach to elucidate the role of LXR α and LXR β in the regulation of renin.

LXRs have been identified as modulators of lipid and glucose metabolism (6–10), inflammation (11–13), and immunity (14, 15). Through several mechanisms, LXRs can also exert significant effects on the cardiovascular system, and their putative antiatherosclerotic properties are of particular therapeutic interest (16). Herein, we describe a novel role of LXR α and LXR β as regulators of renin.

Results

LXR β binds to CNRE. We have previously shown that LXR α can bind to a noncanonical responsive element termed CNRE and thus is able to regulate gene expression in transformed renin-expressing cell lines (2, 3, 17). Based on its high homology and functional overlap with LXR α , we now studied the role of LXR β in this respect. First, we examined the ability of LXR β to bind to CNRE. For this purpose, LXR β was prepared by in vitro transcription and translation and subjected to electrophoretic mobility shift assays (EMSAs). The

Nonstandard abbreviations used: ChIP, chromatin immunoprecipitation; CNRE, overlapping of a cAMP and negative response element; EMSA, electrophoretic mobility shift assay; JG, juxtaglomerular; LXR, liver X receptor; PRA, plasma renin activity; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; RAAS, renin-angiotensin-aldosterone system.

Conflict of interest: Richard Lawn and Jeffrey Chisholm are employees of CV Therapeutics Inc.

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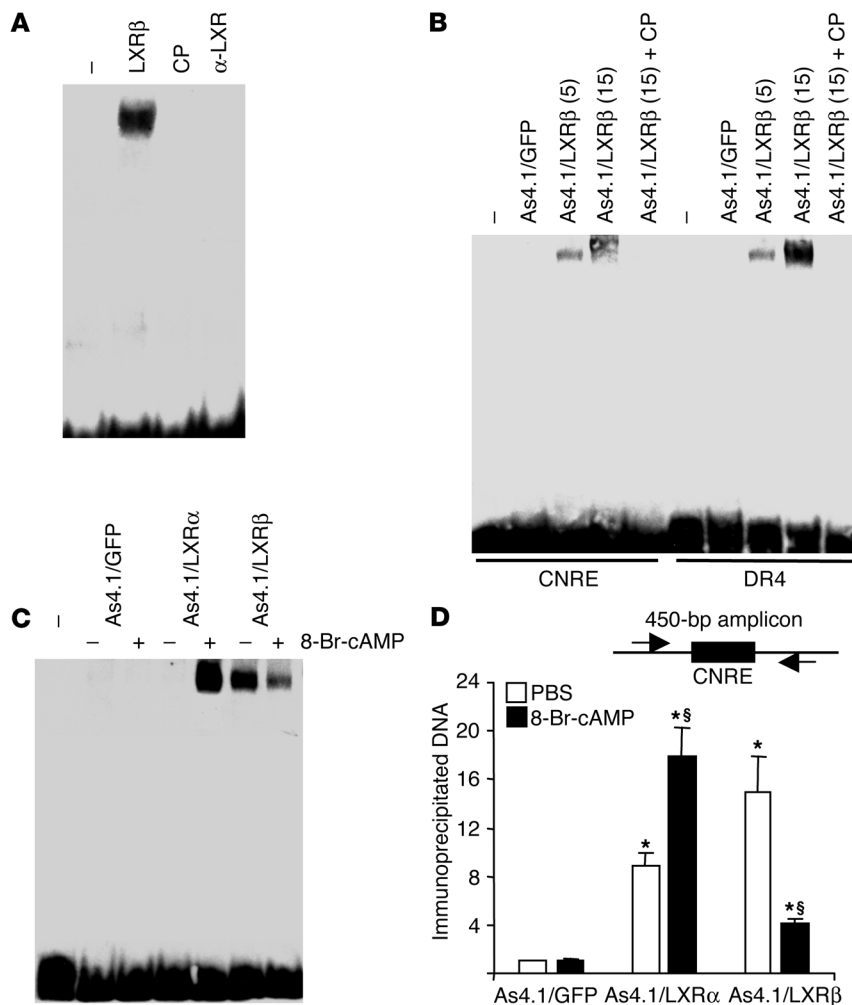


Figure 1
 LXRβ can bind to the CNRE of the *renin* promoter. (A) EMSA of in vitro-transcribed/translated LXRβ binding to a CNRE probe. The shift was not detected after preincubation with excess cold probe (CP) or with an anti-LXR antibody (α-LXR). Dashes indicate lanes where no nuclear extract was added. (B) EMSA of nuclear extracts of As4.1/LXRβ (numbers in parentheses indicate micrograms of nuclear extract) or As4.1/GFP binding to a CNRE or a DR4 probe. The shift was absent in control cells (GFP) not overexpressing LXRβ and was eliminated after preincubation with excess cold probe. (C) EMSA of nuclear extracts of As4.1/GFP, As4.1/LXRα, and As4.1/LXRβ cells binding to a CNRE element after 1 hour of incubation with 1 mM 8-Br-cAMP (+) or vehicle (–). No shift was observed with nuclear extracts of control cells. (D) ChIP assay of As4.1/GFP, As4.1/LXRα, and As4.1/LXRβ cells. Immunoprecipitation of chromatin was performed with an anti-LXR polyclonal antibody, and DNA fragments spanning CNRE were analyzed by qPCR. The amplicon (450 bp) included the CNRE of the *renin* promoter (~600 bp relative to the transcription site). For each condition, the amount of precipitated DNA was normalized to the total DNA input introduced in the ChIP reaction and is presented in relative units compared with untreated As4.1/GFP cells. **P* < 0.05 versus GFP; §*P* < 0.05 versus PBS.

incubation of LXRβ with a labeled CNRE probe produced a shifted band, which was abolished by preincubation with an anti-LXR antibody or with an excess of unlabeled probe (Figure 1A). We next asked whether the binding of LXRβ to CNRE could also be observed in cultured renin-expressing cells. Stable transfectants of As4.1 cells overexpressing LXRβ (As4.1/LXRβ cells) were generated by infection with a bicistronic retroviral vector encoding GFP and LXRβ. As4.1 cells infected with a retroviral vector encoding GFP alone were used as controls (As4.1/GFP cells). RT-PCR and Western blot analyses

showed that As4.1/GFP cells expressed low levels of LXRβ and no LXRα and confirmed the overexpression of LXRβ in As4.1/LXRβ cells (data not shown). The nuclear extract from As4.1/LXRβ and control cells was used in EMSA assays, after incubation with a CNRE- or a DR4-labeled probe. The nuclear extract from As4.1/GFP cells did not produce a shifted band, while the nuclear extract from As4.1/LXRβ cells showed strong binding to the CNRE and the DR4 probe (Figure 1B).

We have previously shown in As4.1 cells that LXRα can regulate gene expression in response to cellular cAMP (2, 3, 17). We therefore asked whether a similar cAMP response was typical of LXRβ as well. Accordingly, we treated As4.1/GFP, As4.1/LXRα, and As4.1/LXRβ cells with 1 mM 8-Br-cAMP or vehicle. The nuclear extracts were obtained 1 hour after treatment and incubated with a labeled CNRE probe. No binding activity was observed in As4.1/GFP cells, either with or without stimulation with cAMP (Figure 1C). cAMP treatment was associated with a strong binding of LXRβ to CNRE in As4.1/LXRα cells, while no binding activity was detected when these cells were untreated. On the contrary, As4.1/LXRβ cells showed binding to CNRE under basal conditions, which was reduced following cAMP stimulation.

LXRs bind to the renin promoter and thus regulate renin gene expression. We used a chromatin immunoprecipitation (ChIP) assay to evaluate the physical interaction of LXRs with the CNRE situated in the *renin* promoter of As4.1 cells. For this purpose, nuclear proteins of As4.1/GFP, As4.1/LXRα, and As4.1/LXRβ cells were cross-linked to DNA after 1 hour of incubation with 1 mM 8-Br-cAMP or vehicle. Following chromatin shearing, immunoprecipitation was performed with an anti-LXR polyclonal antibody, and precipitated DNA fragments were analyzed by quantitative PCR (qPCR). In As4.1/LXRβ cells, the binding of LXRβ to the *renin* promoter was detected under basal conditions and decreased following cAMP stimulation (Figure 1D). Conversely, in As4.1/LXRα cells, LXRα interaction with the *renin* promoter was significantly increased by cAMP stimulation.

We then assessed by quantitative RT-PCR (qRT-PCR) the effect of LXRβ on *renin* mRNA levels and the response to cAMP (Figure 2A). In As4.1/LXRα cells, intracellular cAMP elevation was followed by the upregulation of *renin* mRNA at 6 hours after stimulation. Conversely, the overexpression of LXRβ was associated with higher basal levels of *renin* mRNA, whereas cAMP treatment was followed by a reduction in *renin* mRNA levels. In a time course study, *renin* mRNA levels in As4.1/LXRβ cells reached a minimum 12 hours after treatment with cAMP, when the positive

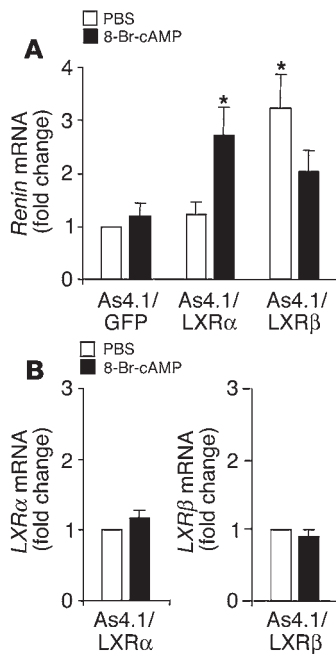


Figure 2

LXR β can regulate *renin* in As4.1 cells. (A) qRT-PCR of *renin* mRNA levels in As4.1 cells overexpressing GFP alone, LXR α , or LXR β after 6 hours of treatment with 1 mM 8-Br-cAMP or vehicle (PBS). (B) Results of qRT-PCR analysis of LXR α and LXR β in As4.1/LXR α and As4.1/LXR β after 6 hours of treatment with 1 mM 8-Br-cAMP or vehicle. mRNA levels were quantified relative to an endogenous control and are presented relative to PBS-treated As4.1/GFP. * $P < 0.05$ versus PBS.

transcriptional effect of LXR β was lost (data not shown). No significant change in LXR α and LXR β levels was detected in treated cells (Figure 2B), which implies that cAMP affects the action of LXRs at the post-transcriptional level. In summary, these findings suggest that LXRs are capable of a complementary effect on *renin* transcription, with LXR β providing a baseline positive regulation on renin expression and LXR α conferring dynamic cAMP responsiveness.

Transcription factors can be functionally regulated through the control of their translocation to the cell nucleus. Therefore, the nuclear localization of LXR α and LXR β with respect to cAMP treatment was assessed with fluorescence immunocytochemistry in As4.1 cells overexpressing N-terminal HA-tagged LXR α (As4.1/HA-LXR α) or LXR β (As4.1/HA-LXR β), which allowed for the detection of tagged proteins with a highly specific antibody (Figure 3). As4.1/HA-LXR cells lines were generated by infection with a retrovirus encoding for HA-LXR α or HA-LXR β . Preliminary studies confirmed that HA-tagged LXRs maintained the biological properties of native LXRs with respect to CNRE binding and renin regulation. Both HA-LXR α and HA-LXR β localized in the nucleus at baseline, and cAMP treatment was not associated with detectable changes in the nuclear localization of HA-LXR α and HA-LXR β . Thus, intracellular trafficking does not appear to be a major mechanism in the regulation of *renin* gene expression by LXR α and LXR β .

LXRs are expressed in JG cells in vivo with a specific enrichment in LXR α . Having established that LXRs can regulate *renin* gene expression with complementary mechanisms in vitro, we next examined their physiological importance in vivo. First, if LXRs play a role in the physiological regulation of renin, they must be expressed in JG cells. Accordingly, we performed double in situ hybridization for LXR α or LXR β and *renin* in the mouse kidney (Figure 4A). As expected, in situ hybridization showed localization of *renin* exclusively in JG cells. Significantly, JG cells were also enriched in LXR α mRNA, displaying the highest levels of LXR α mRNA in the kidney cortex. On the other hand, LXR β appeared to be ubiquitously

expressed in the kidney, without detectable enrichment in the JG apparatus (data not shown). The merging of the images obtained from dual in situ hybridization revealed a strict colocalization of *renin* and LXR α in JG cells. The expression of LXR α in the JG apparatus was confirmed by immunohistochemistry (Figure 4B) using a specific anti-LXR α antibody. As shown in cultured cells, LXR α mainly localized in the cell nucleus.

Independent and corroborating evidence for the colocalization of renin and LXRs was provided by the study of transgenic mice expressing enhanced GFP (EGFP) under the control of the *renin* promoter (18). This strain of mice exhibits EGFP expression exclusively in renin-producing cells. FACS sorting was employed to isolate EGFP-expressing JG cells from the kidneys. RNA isolated from unfractionated kidney cells and from the EGFP⁺ fraction was kindly provided by C.A. Jones and K.W. Gross (Roswell Park Cancer Institute, Buffalo, New York, USA) and was assayed for mRNA levels of *GAPDH*, *renin*, *EGFP*, LXR α , and LXR β (Figure 4C). As expected, both *renin* and *EGFP* mRNA levels were higher in EGFP⁺ cells than in unfractionated kidney cells. Indeed, JG cells showed a significant enrichment in LXR α , while LXR β was expressed at similar levels in both sorted and unsorted cells. Therefore, independent approaches showed that both LXRs are expressed in the kidney in JG cells, with a specific enrichment in LXR α .

Binding of LXRs to the *renin* promoter occurs in vivo and is physiologically regulated. We assessed the interaction of LXRs with the CNRE of the *renin* gene promoter in vivo by ChIP assay. Whole mouse kidneys were disrupted, and nuclear proteins were cross-linked to

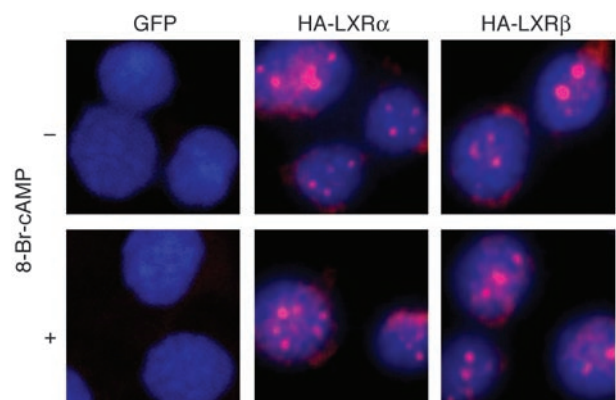


Figure 3

Nuclear localization of LXR α and LXR β is not affected by cAMP elevation. Double fluorescent immunocytochemistry (magnification, $\times 400$) of As4.1 cells overexpressing N-terminal HA-tagged LXR α (HA-LXR α) or LXR β (HA-LXR β) at baseline (top row) and 1 hour after treatment with 1 mM 8-Br-cAMP (bottom row). Control cells expressing GFP alone are also shown. Nuclei were stained with DAPI (blue), and HA-LXR α and HA-LXR β were detected with an anti-HA monoclonal antibody and with a rhodamine-labeled secondary antibody (red).

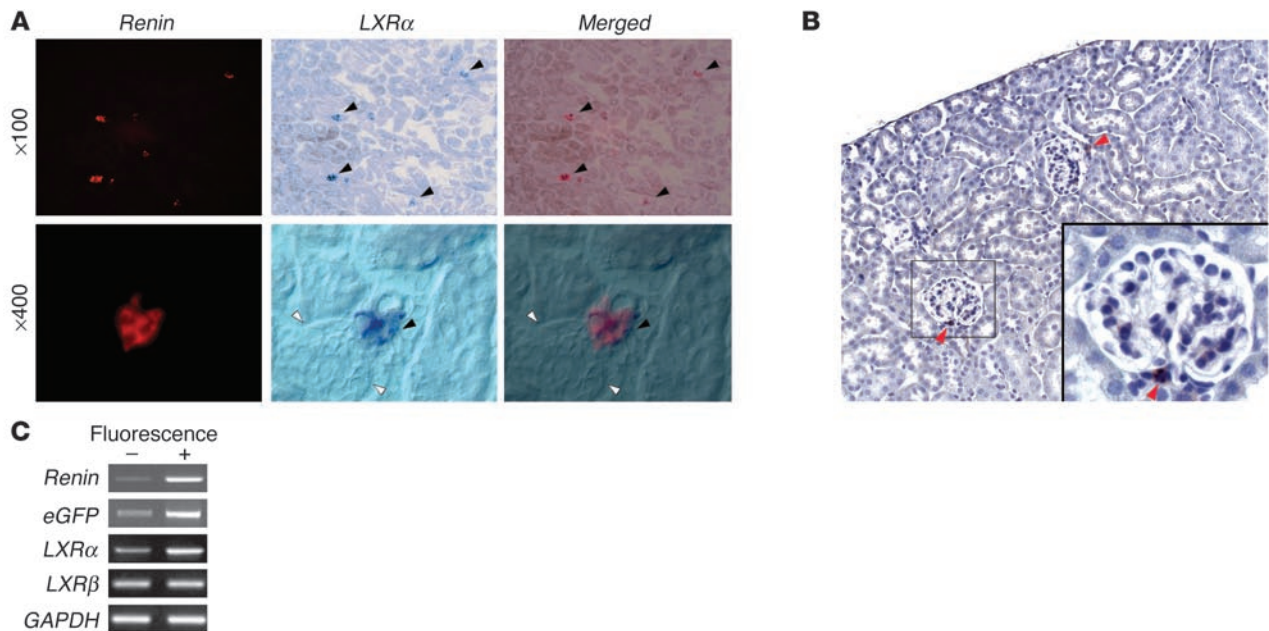


Figure 4

LXRs are coexpressed with renin in JG cells in vivo. (A) Double nonradioactive in situ hybridization of *LXRα* and *renin* in the kidney cortex. *Renin* mRNA was detected with Cy3 (left), and *LXRα* mRNA was detected with BCIP/NBT (middle). Both signals colocalized in the JG apparatus (right). Filled arrowheads indicate juxtaglomerular areas, and open arrowheads indicate the glomerular perimeter. (B) Immunohistochemistry of the kidney cortex (magnification, $\times 200$ and $\times 600$ [inset]). *LXRα* was detected with diaminobenzidine (dark red). Counterstaining was performed with hematoxylin. Arrowheads indicate the positive cells in the JG apparatus. (C) Semiquantitative RT-PCR (38 cycles) for *renin*, *EGFP*, *LXRα*, and *LXRβ* performed on total RNA extracted from the fluorescent (+) and the nonfluorescent (–) fraction of kidneys from transgenic mice harboring an EGFP reporter gene under the control of the *renin* promoter.

DNA with formaldehyde. Immunoprecipitation was performed with an anti-LXR polyclonal antibody. PCR analysis of the recovered DNA fragments showed that LXRs actively bound the *renin* promoter in the region that includes CNRE (Figure 5A).

Next, to examine the in vivo regulation of the LXR-CNRE interaction, we assayed the binding activity to CNRE of kidney nuclear extracts obtained from different physiological conditions of activated renin expression: chronic infusion of β -adrenergic agonist isoproterenol, administration of a hyposodic diet plus the angiotensin converting enzyme (ACE) inhibitor captopril, and clipping of 1 renal artery (modified Goldblatt’s 2 kidney–1 clip model). Each physiological model was associated with an increase in kidney *renin* mRNA and plasma renin activity (PRA; Table 1). However, kidney mRNA levels of *LXRα* and *LXRβ* were unchanged under these conditions compared with those of controls (Figure 5B).

EMSA assays were performed by incubating the nuclear extracts with a labeled CNRE probe (Figure 5C). In renal extracts from control animals receiving a normosodic diet, a modest shift was observed. Of note, each physiological perturbation leading to enhanced renin expression was associated with a dramatic increase in the amount of shifted probe. The shifted band could be eliminated after preincubation with an excess of cold CNRE probe or with an anti-LXR antibody (Figure 5D). These results provide evidence that LXRs do bind to the *renin* promoter in vivo in a regulated fashion. In particular, physiological states of JG activation, which imply cAMP signaling, are associated with increased binding to CNRE. The regulation of kidney *LXRα* and *LXRβ* content does not appear to be involved in this process.

LXR agonists enhance renin gene expression in vivo. We then asked whether a primary activation of LXRs would also result in increased renin expression. For this purpose, a single dose of the LXR agonist T0901317 (50 mg/kg) or vehicle (0.75% carboxymethylcellulose) was administered by gavage to mice receiving a normal diet. The T0901317 compound, a synthetic agonist, has been previously shown to induce in vitro and in vivo the expression of a series of lipid metabolism–related genes through the activation of LXRs (16, 19). The animals were sacrificed at 4, 6, and 8 hours after drug administration, and their visceral organs were harvested for RNA isolation. As expected, the administration of T0901317 resulted in the upregulation of *Abca1* and *Srebp1c* in the bowel, liver, and kidneys (Figure 6A). qRT-PCR analysis showed a concomitant upregulation of *renin* mRNA in the kidneys, which peaked at 6 hours (Figure 6B). Similar results were obtained with a distinct LXR agonist, GW3965 (10 mg/kg; data not shown). These observations provide evidence that a primary ligand-dependent activation of LXRs can result in increased *renin* transcription in vivo.

LXR-null mice show impaired renin regulation. We next examined the renin status of *LXRα*^{–/–}, *LXRβ*^{–/–}, and *LXRα*^{–/–}*LXRβ*^{–/–} mice compared with wild-type littermates (Figure 7). These animals have been described previously (12, 20, 21). In *LXRα*^{–/–} mice, kidney *LXRβ* mRNA was upregulated (2.3-fold compared with wild type; $P < 0.05$), while in *LXRβ*^{–/–} mice, *LXRα* mRNA was downregulated (2.8-fold compared with wild type; $P < 0.05$), consistent with the existence of cross-talk of *LXRα* and *LXRβ* in vivo. When untreated, *LXRβ*^{–/–} and *LXRα*^{–/–}*LXRβ*^{–/–} mice had lower kidney *renin* mRNA levels compared with wild-type and *LXRα*^{–/–}

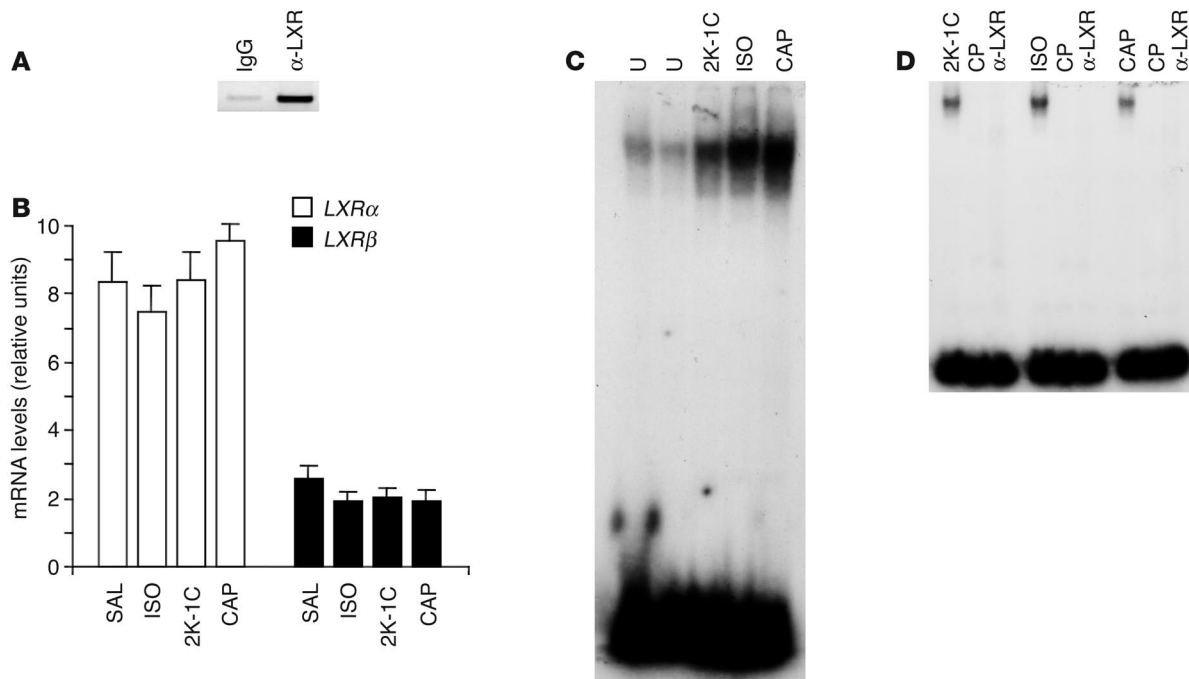


Figure 5

LXR binding to the *renin* promoter occurs in vivo in a regulated fashion. (A) ChIP assay performed on mouse kidneys. Immunoprecipitation of chromatin was performed with an anti-LXR polyclonal antibody or with control IgG antibodies. DNA fragments were analyzed by PCR (40 cycles) and resolved on 1% agarose gel. The amplicon (450 bp) included CNRE of the *renin* promoter (~600 bp relative to the transcription site). (B) qRT-PCR of *LXRα* and *LXRβ* mRNA on total RNA extracted from mice that had undergone 7-day chronic subcutaneous isoproterenol infusion (ISO; 10 mg/kg/d), 7-day monolateral renal artery clipping (modified Goldblatt's 2 kidney–1 clip model [2K-1C]), or 10-day hyposodic diet (0.02% NaCl) plus 5-day oral captopril administration (CAP; 100 mg/kg/d). Control mice underwent saline infusion for 7 days via osmotic minipumps (SAL). *Renin* mRNA levels were quantified relative to an endogenous control. (C and D) EMSAs of nuclear extracts from mice that had undergone monolateral renal artery clipping, ISO, and CAP and from control untreated mice (U, shift from 2 representative animals shown). Ten micrograms of nuclear extract were used per lane. The shift was not detected after preincubation with an excess of cold probe or an anti-LXR antibody.

($P < 0.05$) mice, with an average reduction of 33.8% relative to wild type. A similar trend was observed in PRA (24.5% decrease), although this did not reach statistical significance.

The functional response of JG cells was then examined following β -adrenergic stimulation, which directly implies cAMP signaling in JG cells. For this purpose, kidney *renin* mRNA levels and PRA were measured after 7 days of continuous β -adrenergic stimulation (5 mg/kg/d isoproterenol in 0.9% saline via a subcutaneous osmotic pump). Control mice received a chronic infusion of 0.9% saline alone. As a homeostatic response to pump implantation and saline infusion alone, *renin* mRNA significantly dropped in wild-type mice (54%; $P < 0.05$) compared with untreated wild-type mice, while only a minor additional reduction was observed in saline-infused *LXRβ*^{-/-} and *LXRα*^{-/-}*LXRβ*^{-/-} mice compared with untreated mice (14% and 33% respectively; NS). Thus, no significant difference in *renin* mRNA among wild type, *LXRβ*^{-/-}, and *LXRα*^{-/-}*LXRβ*^{-/-} was observed with saline infusion. *Renin* mRNA was also unchanged in saline-infused compared with untreated *LXRα*^{-/-} mice. In wild-type mice, isoproterenol-saline administration was followed by a 2.6-fold upregulation ($P < 0.05$) of *renin* mRNA and a parallel 2.0-fold induction of PRA ($P < 0.05$) compared with levels in saline-treated controls. A similar response was observed in *LXRβ*^{-/-} mice (*renin* mRNA, 1.8-fold and PRA, 1.9-fold; $P < 0.05$). Strikingly, the chronic infusion of the adrenergic agonist isoproterenol did not produce any increase in *renin* mRNA

levels and PRA in *LXRα*^{-/-} and *LXRα*^{-/-}*LXRβ*^{-/-} mice ($P < 0.05$ versus wild-type and *LXRβ*^{-/-}), consistent with a key role of *LXRα* in the functional response of the JG apparatus to the β -adrenergic/adrenyl-cyclase signaling pathway.

In summary, the absence of *LXRα* was associated with the abolishment of the β -adrenergic/cAMP-dependent activation of the JG system in vivo. Also, lower *renin* mRNA levels were detected in untreated *LXRβ*^{-/-} and *LXRα*^{-/-}*LXRβ*^{-/-} mice, while *LXRα*^{-/-} mice, presenting an upregulation of kidney *LXRβ*, were unable to downregulate *renin* when infused with saline, which suggests that *LXRβ* participates in the regulation of basal *renin* transcription in vivo.

Discussion

LXRs are emerging players in the pathophysiology of the cardiovascular system. However, the involvement of LXRs in the control of salt-volume homeostasis and blood pressure has not been reported before. We show here that, through binding to the *renin* promoter, LXRs positively enhance the transcription of *renin*. In particular, *LXRα* expression is specifically enriched in kidney JG cells in vivo and acts as a key molecule in the chronic activation of *renin* release by adrenergic stimulation. On the other hand, our data suggest that *LXRβ* contributes positive effect under basal conditions on *renin* transcription in vivo but is not strictly required for the functional responses of the JG apparatus.



Table 1
PRA values obtained in the different models

Treatment group	PRA (ng Angl/ml/h)	Treatment group	PRA (ng Angl/ml/h)	Treatment group	PRA (ng Angl/ml/h)
Saline	2,615 ± 1,234	Normal diet	4,713 ± 2,066	Sham operation	1,605 ± 406
Isoproterenol	8,690 ± 1,226 ^A	Salt-restricted diet plus captopril	24,173 ± 2,902 ^A	2K-1C	3,105 ± 1,934

Saline (0.9% NaCl) or isoproterenol (10 mg/kg/d in saline) were administered with a miniosmotic pump for 7 days. For the low salt-captopril treatment, animals were fed a low-salt diet (0.02% NaCl) for 10 days. During the last 5 days, captopril was administered in drinking water (100 mg/kg body wt/d, dissolved in 2% sucrose). Control animals received the usual chow (0.6% NaCl) and 2% sucrose drinking water. For the Goldblatt's 2 kidney–1 clip mouse model (2K-1C) of renal artery constriction, a U-shaped clip (0.12-mm opening width) was placed on the renal artery close to the aorta for 7 days. Sham-operated animals underwent the same operation without clip placement. Angl, angiotensin I. ^A*P* < 0.05 versus respective control.

Transcriptional regulation is a major step in the complex control of renin production and release from JG cells, the principal source of circulating renin (1). The regulatory sequences of the *renin* gene include a proximal promoter and additional regulatory elements located further upstream, mostly in areas of substantial interspecies homology (22, 23). Several transcription factors have been described as potential regulators of renin expression in vitro. These include NF-κB (24), CREB/CREM (25), vitamin D receptor (26), HOX/PBX (27), and many others. However, because the primary culture of JG cells is very difficult due to a rapid decrease of renin expression and a loss in cellular phenotype, most of the evidence is based on in vitro studies of cultured cell lines, only some of them constitutively expressing renin. These include As4.1 and Calu-6 cells. The latter are derived from a renin-expressing human lung anaplastic carcinoma (28), while As4.1 is a transformed cell line obtained from a mouse kidney tumor (29). As4.1 cells constitutively express, package, and secrete renin, but they do not express LXRα and are unable to respond to cAMP stimulation with increased renin transcription, a fundamental ability of JG cells in vivo (2). Despite providing a convenient and high-throughput in vitro model for the study of renin synthesis and release, these cell lines may not completely share the biological properties of JG cells and are obviously deprived of the functional microenvironment of the JG apparatus. Therefore, a full understanding of the physiological regulation of renin by any molecular mechanism requires additional and complementary in vivo evidence.

Previous data showed that CNRE, an overlapping of a cAMP response element and a negative response element found in the promoter of several genes including *c-myc* and *renin* (mouse, rat, human), may be involved in the tissue-specific expression of renin (30). By binding to this element and not to DR4 elements, LXRα can function as a cAMP-responsive ligand-independent transcription factor in As4.1 cells and Calu-6 cells (2, 3, 17). Based on the current observations, LXRβ, a highly homologous nuclear receptor, is also able to bind to CNRE and thus upregulate renin expression. However, treatment with cAMP resulted in dissociation of LXRβ from the *renin* promoter and reduced affinity for CNRE. Therefore, the current study provides evidence of a novel dissociation of LXRα from LXRβ functions via a specific responsive element. This phenomenon is possibly related to differences in regulatory regions of these nuclear receptors such as phosphorylation sites.

The present in vivo observations provide physiological significance to and perspective on the in vitro evidence. We show here that LXRα and LXRβ do physically interact in the kidney with the CNRE of the *renin* gene promoter. Also, the binding of LXRs to CNRE paralleled the functional status of the JG apparatus.

In fact, physiological manipulations that enhance renin expression (chronic β-adrenergic treatment, renal artery clipping, low-salt diet combined with ACE inhibition) resulted in a dramatic increase in binding activity to CNRE of kidney nuclear extracts. Given the unchanged expression of LXRs, this observation suggests that LXRα is activated under conditions of high renin expression, possibly through a cAMP/PKA pathway. In particular, the elevation of renin by isoproterenol infusion essentially relies on the triggering of the cAMP signaling cascade by the adenylyl cyclase-coupled β-adrenergic receptor.

Importantly, the upregulation of renin and PRA following isoproterenol infusion was completely abolished in *LXRα*^{-/-} and *LXRα*^{-/-}*LXRβ*^{-/-} mice. This loss-of-function phenotype underscores the key role of LXRα as a cAMP-responsive factor and its critical function in the regulation of the JG apparatus by the adrenergic system. Interestingly, CNRE is not the only cAMP response ele-

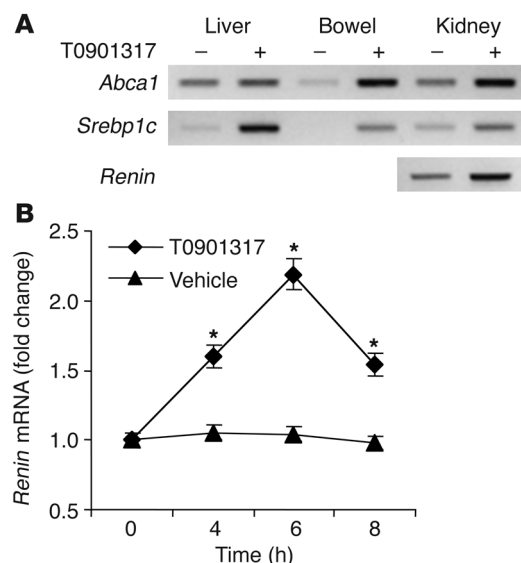
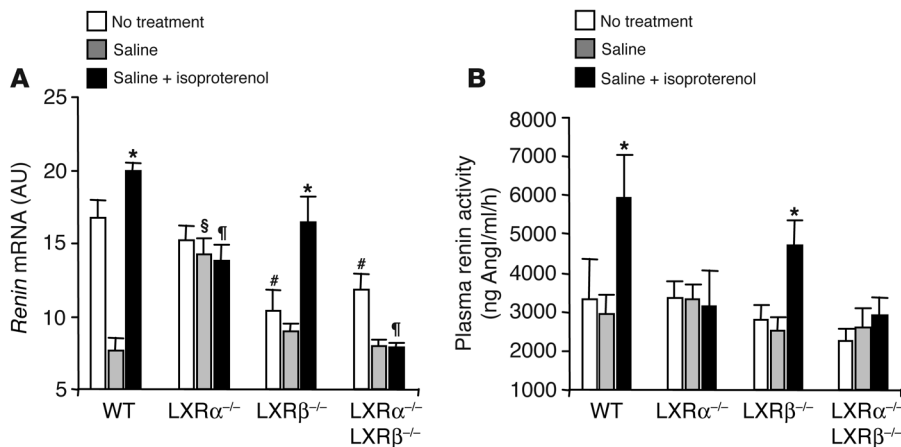


Figure 6
LXR agonism acutely upregulates *renin* in vivo. (A) Semiquantitative RT-PCR (35 cycles) for LXR-regulated genes *Abca1*, *Srebp1c*, and *renin* on total RNA extracted from liver, bowel, and kidney of mice 6 hours after the oral administration of LXR agonist T0901317 (50 mg/kg) or vehicle (0.75% carboxymethylcellulose). Bands were resolved on a 1% agarose gel. (B) qRT-PCR of *renin* mRNA levels in kidneys obtained from mice 0, 4, 6, and 8 hours after the administration of T0901317 or vehicle. *Renin* mRNA levels were quantified relative to an endogenous control. **P* < 0.05 versus vehicle.

**Figure 7**

Kidney *renin* mRNA and PRA of wild-type, *LXR $\alpha^{-/-}$* , *LXR $\beta^{-/-}$* , and *LXR $\alpha^{-/-}$ LXR $\beta^{-/-}$* mice. **(A)** Kidney *renin* mRNA in untreated mice (white bars) and in mice that had undergone 7-day subcutaneous infusion of saline (gray bars) or isoproterenol (5 mg/kg/d in saline; black bars) via an osmotic minipump. *Renin* mRNA levels were measured by qRT-PCR on total kidney RNA and are expressed in AU. * $P < 0.05$ versus saline; § $P < 0.05$, ¶ $P < 0.05$, and # $P < 0.05$ versus the other strains. **(B)** PRA of the same treatment groups. AngI, angiotensin I. * $P < 0.05$ versus saline-treated mice.

ment that has been characterized in the *renin* promoter. Two other cAMP response elements have been described in the mouse *renin* gene. One of them is a CRE element located in the *renin* enhancer (25), and the other is a HOX/PBX site (RP-2 element) in the proximal promoter (27). However, these elements do not confer full cAMP responsiveness to the *renin* gene in As4.1 cells (2, 31). According to the current observations in *LXR $\alpha^{-/-}$* and *LXR $\alpha^{-/-}$ LXR $\beta^{-/-}$* mice, these elements alone do not appear to be sufficient for the upregulation of *renin* by cAMP in vivo.

LXR $\beta^{-/-}$ mice exhibited a normal *renin* response to adrenergic stimulation, a result that rules out a primary role of *LXR β* in this respect. However, untreated *LXR $\beta^{-/-}$* and *LXR $\alpha^{-/-}$ LXR $\beta^{-/-}$* animals presented basal *renin* mRNA levels approximately 35% lower than those of their wild-type littermates. Conversely, *LXR $\alpha^{-/-}$* animals, which exhibited higher kidney *LXR β* levels, failed to downregulate *renin* mRNA levels when infused with saline. In vitro, *LXR β* was able to bind to the *renin* promoter and upregulated baseline *renin* expression in cultured cells. Together, these findings suggest that *LXR β* participates in the baseline regulation of *renin* transcription in vivo but is not strictly required for *renin* expression. However, a number of potential limitations should be considered. First, we only observed a nonsignificant trend toward lower PRA (by 25%) in *LXR $\beta^{-/-}$* and *LXR $\alpha^{-/-}$ LXR $\beta^{-/-}$* mice, possibly due to the limited power of the study to detect small changes in PRA. Second, following saline infusion, we did not detect significantly lower *renin* mRNA levels in *LXR $\beta^{-/-}$* and *LXR $\alpha^{-/-}$ LXR $\beta^{-/-}$* mice, a finding possibly related to the concomitant downregulation of *renin* mRNA observed in wild-type mice. Thus, the actual physiological contribution of *LXR β* to the maintenance of *renin* status deserves further study. Finally, in vitro, the positive transcriptional effect of *LXR β* was diminished by cAMP elevation, resulting in a downregulation of *renin* mRNA in As4.1/*LXR β* cells. In vivo, we detected only a minor and not statistically significant reduction in *renin* mRNA and PRA in *LXR $\alpha^{-/-}$* mice treated with isoproterenol. We do not have a direct explanation for this result. It is likely that in vivo, other factors may compensate for the reduction of *LXR β*

action during isoproterenol stimulation or that the chronic activation of *LXR* signaling may involve pathways not explored in single 8-Br-cAMP stimulation experiments and that require further elucidation.

Increasing evidence is accumulating regarding the role of *LXRs* in atherosclerosis (16, 32–34). In particular, their beneficial effects on cholesterol metabolism, atherogenesis, and insulin sensitivity have suggested that pharmacological targeting of *LXRs* with synthetic agonists may provide a new therapeutic strategy for prevalent cardiovascular diseases (35, 36). So far, one obstacle for the implementation of this pharmacological approach has been the important lipogenic effect of these drugs, which leads to liver steatosis and hepatocellular damage (37, 38). In our experimental context, a single administration of *LXR* agonists was associated with a slight but significant

increase in *renin* transcription. Whether a longer treatment with *LXR* agonists would result in a persistent upregulation of *renin* transcription and ultimately in a full activation of the RAAS at the plasma or tissue level requires further investigation. However, the documented dissociation of *LXR α* and *LXR β* function with respect to CNRE signaling underscores the potential of selective *LXR* modulation. Based on our data, we speculate that selective pharmacological activation of *LXR β* in JG cells may in fact lead to a favorable inhibition of the RAAS, in addition to the other metabolic effects of *LXR* agonism. Thus, we speculate that novel *LXR*-targeted drugs might have the pleiotropic potential to increase reverse cholesterol transport, reduce endogenous cholesterol biosynthesis, and exert antiatherosclerotic effects, while also reducing the activity of the RAAS.

The identification of *LXR α* and *LXR β* as regulators of *renin* has important implications. Recently, Daugherty et al. have shown that hypercholesterolemia is associated with increased levels of circulating angiotensinogen and angiotensin peptides and that all the components of the RAAS, including *renin*, are overexpressed within atherosclerotic lesions (39). Of note, *LXR α* is physiologically activated during cholesterol loading (12), and the expression and activation of *LXR α* inside the atherosclerotic plaque have also been described (34). Taken together, several lines of evidence suggest that *LXR α* and other nuclear hormone receptors may in fact represent major mediators of a cross-talk among lipid metabolism disorders, the RAAS, and blood pressure regulation, both throughout the cardiovascular system and in the kidney.

Also, we have previously reported that *LXR α* can upregulate *c-myc* and other genes involved with cell proliferation and differentiation (2, 3, 40). Thus, *LXRs* may be important transcription factors mediating JG cell hyperplasia in pathological conditions. A better understanding of these novel functions of *LXRs* will provide valuable information for the development of novel selective modulators of *LXR α* and/or *LXR β* function for several disorders including hypertension, heart failure, and other pathological conditions associated with activation of the RAAS.



Methods

Cell culture. The mouse renin-expressing cell line As4.1 (catalog no. CRL2193; ATCC) was previously isolated from the kidneys of transgenic animals harboring a chimeric renin gene promoter/SV40 T antigen construct (29). Cells were cultured in high-glucose DMEM (Invitrogen Corp.) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin) at 37°C in 5% CO₂. For cAMP stimulation, cells were first made quiescent in DMEM containing 0.1% fetal calf serum for 12 hours. Fresh medium was added containing DMEM with 0.1% fetal calf serum plus either vehicle (PBS) or 1 mM 8-Br-cAMP, and cells were incubated for different amounts of time. For the generation of As4.1 cell lines stably expressing GFP, LXR α , LXR β , HA-LXR α , and HA-LXR β cDNAs were cloned into a MSCV-IRES-GFP plasmid backbone (a sequence encoding for the HA tag was added by PCR immediately 5' of the start codon, in frame with the coding sequence). Retroviral particles were then obtained by tripartite transfection in HEK 293 T cells and concentrated by ultracentrifugation. Each cell line was generated by infection of 10⁷ cells with an average of 10⁶ viral particles in the presence of Polybrene (Sigma Aldrich). This resulted in an infection rate more than 90%. Pools of infected cells were then subcultured.

RNA analysis. Total RNA was extracted with TRIzol reagent (Invitrogen Corp.) or with affinity resin columns (QIAGEN). First-strand cDNA was prepared using oligo-dT priming and subsequently used (200 ng per reaction) as a template for semiquantitative RT-PCR or qRT-PCR. qRT-PCR was performed with TaqMan technology on an ABI Prism 7700 Applied Biosystems sequence detection system. mRNA levels were expressed relative to an endogenous control or in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. qRT-PCR primers used were: *REN1c* (sense, CCCTCCCCGACATTTCCT; antisense, GCACGTAGTCCGTACTGCTGAGT; probe, TGACCTGGGAGGCAGGGCCTACA) and *GAPDH* (Assays-On-Demand; Applied Biosystems). Semiquantitative RT-PCR primers used were: *REN1c* (sense, ACCTTCAGTCTCCCAACACG; antisense, GAAGGGGCAAACACTCGTTAA), *LXR α* (sense, GGATAGGGTTGGAGTCAGCA; antisense, GGAGC-GCCTGTTACACTGTT), *LXR β* (sense, GCTCAGGAGCTGATGATCCA; antisense, GCGCTTGATCCTCGTGTAG), *EGFP* (sense, AGCAAGGGC-GAGGAAGTGTCTACTG; antisense, GGTGGACAGGTAATGGTTGTCTGGG), *GAPDH* (sense, ATGGTGAAGGTCGGTGTG; antisense, ACCAGTGGATGCAGGGAT), *Abca1* (sense, AGTACCCAGCCTGACACTT; antisense, ACGTGTCTTGCTCAGCTTC), *Srebp1c* (sense, GTTACTC-GAGCCTGCCTTCAGG; antisense, CAAGCTTTGGACCTGGGTGTG). Bands were resolved on 1% agarose gels stained with ethidium bromide.

In vitro transcription and translation. In vitro-transcribed and -translated LXR β was prepared with a PCR-based system (RTS *E. coli*; Roche Diagnostics Inc.) using first-strand mouse kidney cDNA as a template. To the coding sequence of interest, regulatory elements (T7 promoter, ribosomal binding site, and T7 terminator) were added by PCR as recommended by the manufacturer. Primers for the first PCR reaction were: sense, CGCTTAATTAACATATGACCTCTTCCCCACAAGTTCTCT; antisense, TTAGTTAGTTACCGGATCCCTTACTACTCGTGCATCCAGA. The obtained template DNA was added to the reaction solution (0.1 μ g/reaction) containing *E. coli* lysate and incubated at 30°C for 6 hours under continuous shaking.

EMSA. EMSAs were performed with ³²P-labeled or with digoxigenin-labeled double-stranded oligonucleotide probes (Roche Diagnostics Corp.). Binding reactions were performed for 15 minutes at room temperature in 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.2% (wt/vol) Tween-20, 30 mM KCl, 5 ng/ μ l poly-L-lysine, 0.15 μ g/ μ l poly d(I-C), 20 fmol labeled double-stranded oligonucleotides, and purified proteins or nuclear extracts (10 μ g each). For competition and neutralization studies, samples were coincubated with a 100-fold

molar excess of unlabeled probe or with anti-LXR α goat polyclonal antibody (Santa Cruz Biotechnology Inc.) for an additional 15 minutes. In preliminary experiments, this antibody showed no LXR isoform specificity and reacted with both murine LXR α and LXR β . After incubation, samples were separated on a native 6% polyacrylamide gel in \times 0.5 tris-borate-EDTA. Nonradioactive samples were subsequently blotted on a nylon membrane, detected through a chemiluminescent reaction with alkaline phosphatase-linked Fab fragments and visualized by autoradiography. For ³²P-labeled probes, the gel was dried and exposed to autoradiography film overnight at -80°C. The nucleotide sequence of the sense oligonucleotide probes were: CTAACCTGGTCTCACAGGCTAGAA (CNRE) and AAAGGGTTTAAATAAGTTCATCCA (DR4).

ChIP. Proteins were cross-linked to DNA in 1% formaldehyde for 10 minutes. For ChIP assays on tissues, kidneys were quickly disrupted with a rotor-stator homogenizer and immediately incubated with formaldehyde. After washing, cells were lysed in detergent lysis buffer. Lysates were then washed and sonicated to an average chromatin length of 500–1,000 bp. After pre-clearing, 5 μ g of antibody was added, and the sample was incubated overnight. Immune complexes were recovered with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology Inc.). After washing, cross-links were removed via incubation at 65°C for 4 hours, and DNA was isolated with resin columns (QIAGEN). Ten percent of the purified DNA was analyzed by PCR (40 cycles) or qPCR, using primers designed to amplify the *renin* gene promoter (sense, TGACCAGGTCCTGCTTATCC; antisense, GCTTGCCCTCCTCTCTCTCT). PCR products were visualized by agarose gel electrophoresis. qPCR was performed with SYBR Green (Applied Biosystems) on an ABI Prism 7700 Applied Biosystems sequence detection system. In order to control for loading differences, the amount of precipitated DNA was normalized to the amount of DNA in the original cell lysate subjected to ChIP.

Genetically modified mice. LXR α ^{-/-}, LXR β ^{-/-}, and LXR α ^{-/-}LXR β ^{-/-} mice were generated as described previously (33). The production of mice expressing EGFP under the control of the *renin* promoter was described previously (18). These mice harbor a reporter construct containing 4.1 kb of the 5'-flanking sequence of renin (-4,100 to -117) fused to EGFP cDNA. The expression of EGFP in the kidney is restricted to the JG cells. For isolation of JG cells, kidneys from male mice were harvested and dissociated by trypsin, and EGFP⁺ cells were collected by cell sorting. RNA extracted from EGFP⁻ and EGFP⁺ fractions was kindly provided by C.A. Jones and K.W. Gross (Roswell Park Cancer Institute, Buffalo, New York, USA).

Animal procedures. All animal procedures were approved by the Harvard University, Karolinska Institutet, and CV Therapeutics Inc. Institutional Animal Care and Use Committees. Four- to 8-week-old C57BL/6 mice were maintained on a 12-hour light/12-hour dark cycle at an ambient temperature of 24°C and 60% humidity. Food and water were provided ad libitum. For all in vivo experiments, each treatment group included 6–8 mice. LXR ligand-treated tissues from male C57BL/6 mice were provided by CV Therapeutics Inc. LXR ligands were administered by gavage in 0.75% carboxymethylcellulose, 0.1% Tween-80, and tissues were obtained by autopsy at the indicated time points. For isoproterenol infusion, animals were implanted with subcutaneous minipumps (ALZET; DURECT Corp.) delivering isoproterenol (5–10 mg/kg/d) in saline solution (0.9% NaCl). Control animals received saline alone. Seven days after pump placement, the animals were euthanized and tissues harvested. For the low-salt/captopril treatment, animals were placed on a low-salt diet (0.02% NaCl) for 10 days. During the last 5 days, captopril was administered in drinking water (100 mg/kg body wt/d, dissolved in 2% sucrose). Control animals received the usual chow (0.6% NaCl) and 2% sucrose drinking water. For the Goldblatt mouse model of renal artery constriction, animals were anesthetized, the left kidney was exposed, and a U-shaped stainless steel clip (3 \times 2 \times 1 mm with a



2-mm-long cleft and 0.12-mm opening width) was placed on the renal artery close to the aorta. Sham-operated animals underwent the same operation without clip placement. Mice received the usual chow and free access to water and were euthanized after 7 days. Before tissue harvesting, animals were anesthetized, the heart was cannulated, and tissues perfused with ice-cold PBS. All collected tissues were stored at -80°C until further use.

In situ hybridization. Dual-probe nonisotopic in situ hybridization was performed as described previously (41). Briefly, cryostat sections were cut to 10- μm thickness, fixed in 4% paraformaldehyde, and acetylated. *LXR α* and *LXR β* probes were 564 and 790 bp, respectively. Hybridization was performed in hybridization buffer with 50% formamide, 5 \times SSC, 2% blocking reagent (Roche Diagnostics Corp.), 0.02% SDS, 0.1% sarcosine, and 100 ng/ml digoxigenin or FITC-labeled cRNA probe at 70°C for 72 hours. After washing, detection was performed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and HRP-conjugated anti-FITC antibodies (DakoCytomation), biotinylated tyramide (TSA reaction assay; PerkinElmer), 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium, and streptavidin-cyanine 3. After rinsing and mounting, light field and fluorescent photographs were taken using a Nikon E600 microscope and a SPOT digital camera (Diagnostic Instruments Inc.). Control sections were obtained with an identical concentration of the sense probe transcripts.

Immunohistochemistry. Immunostaining of paraffin-embedded mouse kidney sections (5 μm thick) was performed using standard procedures. Briefly, the rehydrated tissue sections were subjected to antigen unmasking (DakoCytomation) at 95°C for 20 minutes and endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Subsequently, mouse IgG blocking was performed (M.O.M. reagent; Vector Laboratories), and the sections were incubated overnight with a primary mouse monoclonal anti-LXR α antibody (R&D Systems) and then with a secondary biotinylated anti-mouse IgG antibody. The sections were subsequently incubated with avidin-biotinylated HRP complex (Vector Laboratories) and exposed to 0.1% diaminobenzidine tetrahydrochloride and 0.02% hydrogen perox-

ide as a source of peroxidase substrate. Each section was counterstained with hematoxylin, dehydrated through graded alcohols to xylenes, and mounted with VectaMount (Vector Laboratories). For negative controls, the primary antibody was replaced by 5% BSA.

PRA. PRA was measured as described previously with a radioimmunoassay that detects the amount of angiotensin I produced per hour in the presence of excess angiotensinogen (nanograms of angiotensin I produced per milliliter of plasma per hour) (42). This assay measures the enzymatic activity of active plasma renin in the presence of an exogenous excess of its substrate.

Statistical analysis. Data are reported and represented as mean \pm SE. Statistical analysis was performed using a 2-tailed Student's *t* test and ANOVA with Bonferroni post-hoc analysis. $P < 0.05$ was considered statistically significant.

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