Side Chain-oxidized Oxysterols Regulate the Brain Renin-Angiotensin System through a Liver X Receptor-dependent Mechanism

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Disturbances in cholesterol metabolism have been associated with hypertension and neurodegenerative disorders. Because cholesterol metabolism in the brain is efficiently separated from plasma cholesterol by the blood-brain barrier (BBB), it is an unsolved paradox how high blood cholesterol can cause an effect in the brain. Here, we discuss the possibility that cholesterol metabolites permeable to the BBB might account for these effects. We show that 27-hydroxycholesterol (27-OH) and 24S-hydroxycholesterol (24S-OH) up-regulate the renin-angiotensin system (RAS) in the brain. Brains of mice on a cholesterol-enriched diet showed up-regulated angiotensin converting enzyme (ACE), angiotensinogen (AGT), and increased JAK/STAT activity. These effects were confirmed in in vitro studies with primary neurons and astrocytes exposed to 27-OH or 24S-OH, and were partially mediated by liver X receptors. In contrast, brain RAS activity was decreased in Cyp27a1-deficient mice, a model exhibiting reduced 27-OH production from cholesterol. Moreover, in humans, normocholesterolemic patients with elevated 27-OH levels, due to a CYP7B1 mutation, had markers of activated RAS in their cerebrospinal fluid. Our results demonstrate that side chain-oxidized oxysterols are modulators of brain RAS. Considering that levels of cholesterol and 27-OH correlate in the circulation and 27-OH can pass the BBB into the brain, we suggest that this cholesterol metabolite could be a link between high plasma cholesterol levels, hypertension, and neurodegeneration.

As in several other tissues, the brain expresses a complete and functional renin-angiotensin system (RAS), which has important biological and neurological activities (1). In addition to the established functions in salt and water homeostasis and the regulation of blood pressure, RAS regulates multiple brain functions such as learning and memory, processing of sensory information, and regulation of emotional responses (2). The precursor of angiotensin, angiotensinogen (AGT), is synthesized by glial cells, mainly astrocytes but also by neurons (3). The action of Renin on AGT generates the inactive decapptide, angiotensin I (AngI), which is further converted into angiotensin II (AngII) in a rate-limiting step in the RAS pathway by angiotensin converting enzyme (ACE). AngII mediates its effects by acting on angiotensin II type 1 and 2 receptors (AT1R and AT2R). These receptors are implicated in mechanisms of neuronal plasticity, learning, and memory (4), as well as in the control of cerebral blood flow (5). The physiological effects of AngII are mainly mediated by AT1R, and include vasoconstriction and vasopressin release in the brain (6). Dysregulation of brain RAS has been suggested to be of importance in several pathological processes such as stroke, depression, and neurodegenerative disorders including Alzheimer disease (AD) (7).

Disturbances in cholesterol metabolism and hypercholesterolemia may also play a role in neuropathological conditions (8). The slow conversion of brain cholesterol into the oxyysterol 24S-hydroxycholesterol (24S-OH) maintains the homeostasis of cholesterol in the central nervous system (CNS). This compound passes the blood-brain barrier (BBB) and its outflux is considered to be the major pathway for the excretion of excess cholesterol out of the CNS (9). Another oxyysterol, 27-hydroxycholesterol (27-OH), mainly formed in the periphery is capable of passing into the brain. Plasma levels of cholesterol and 27-OH have been shown to correlate and high levels of cholesterol in the blood result in an increased flux of 27-OH into the brain. In addition, excessive levels of 27-OH have been associated with AD and other neurodegenerative processes (10).

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In the present paper, we present in vivo and in vitro evidence showing that a high cholesterol diet, 27-OH, and 24S-OH activate brain RAS. Our results suggest that one of the biological functions of these side-chain oxidized oxysterols is to modulate RAS in the brain, constituting a mechanistic link between hypertension and hypercholesterolemia that could be of importance in neurodegeneration.

**EXPERIMENTAL PROCEDURES**

*Animals and Experimental Design*—Five- to six-weeks-old mice (strain C57BL/6) were purchased from B&K (Sollentuna, Sweden). The animals were housed in groups of five with a 12-h light/dark cycle. They were fed either a normal chow diet (ND) or a high-fat diet (HFD) containing 21% fat and 0.15% cholesterol (R638, Lactamine, Sweden) for 9 months. Four animals per group were sacrificed by decapitation and the brains immediately frozen on dry ice and stored at −80 °C. Each brain was homogenized in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100) containing protease inhibitor and phosphatase inhibitor mixtures (Sigma).

The generation and breeding of Cyp27a1 knock-out (Cyp27KO) mice has been described previously (11). Cyp27KO mice were fed normal chow and sacrificed at 42 weeks of age. Hippocampal and cortical areas from four animals per group were dissected and immediately frozen on dry ice. Homogenization and processing of samples were done as formerly described. Ethical consent was received from the Karolinska Institutet regional ethical committee.

*Cell Cultures*—Primary cultures of rat neurons and astrocytes were performed as previously described (12, 13). Treatments with 24S-OH, 27-OH, or the liver X receptor (LXR) agonist TO-901317 were done at 1 μM for 24 h. Blocking of LXR was done by preincubation of cells for 3 h with 22(S)-OH (10 μM). In the experiments with the AT1R antagonist, losartan (1 and 10 μM), preincubation was done 30 min before oxysterol treatments. Both oxysterols were obtained from Steraloids. Losartan, 22(S)-OH, and TO-901317 were purchased from Sigma. Ethical consent for experiments with primary cultures was received from the regional ethical committee of Karolinska Institutet.

*Immunoblotting*—Protein levels were quantified using the BCA protein assay kit (Pierce). Equal amounts of protein were separated using 10% acrylamide gel, and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). The blots were incubated with antibodies against AGT (Abbiotec), AT1R (Abbiotec), angiotensin I/II (N-10, Santa Cruz Biotechnology), p-STAT3 (Tyr-705) (Santa Cruz Biotechnology), NeuN (Chemicon International), or actin (Sigma). Incubations with primary antibodies were performed overnight at 1:1000 dilution, followed by incubations with anti-rabbit, anti-mouse, or anti-goat IgG at 1:2000 dilution (Amersham Biosciences). Immunoreactivity was detected by the ECL detection system (Amersham Biosciences). Some immunoblots were stripped using Restore™ Western blot stripping buffer (Pierce) at room temperature for 15 min, and then re-blotted with other antibodies. The relative density of the immunoreactive bands was calculated from the optical density (OD) of the selected area using ImageJ version 1.37 software (NIH).

*RNA Extraction and Real-time RT-PCR*—Total RNA was extracted following the manufacturer’s instructions using the RNAeasy lipid tissue mini kit in the case of mouse brain or RNAeasy mini kit for rat primary neurons and astrocytes, both acquired from Qiagen. Total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time RT-PCR amplification assays for gene targets were performed with a total volume of 20 μl in each well containing 10 μl of PCR Master Mix (Applied Biosystems), 2 μl of cDNA corresponding to 10 ng of RNA, and 1 μl of each TaqMan® Gene Expression Assays (Applied Biosystems). 36B4 and GAPDH mRNA were used as endogenous controls in mice and in vitro experiments, respectively. The relative quantification of all targets were carried out using the comparative cycle threshold method, $2^{-\Delta\Delta C_t}$, where $\Delta C_t = (C_{t,target gene} - C_{t,endogeneous control\_treated} - (C_{t,target gene} - C_{t,endogeneous control\_untreated})$ (14). Each sample was measured in triplicate. Relative transcription levels ($2^{-\Delta\Delta C_t}$) were expressed as a mean ± S.E.

*LXR-β Knockdown by Small Interference RNA Transfection*—Small interference RNA (siRNA) for LXRβ were designed by Dharmacon. Rat primary cultures (80% confluency) were transfected with LXRβ siRNA (ON-TARGET plus SMARTpool; catalogue number L-088852-01-0020) to a final concentration of 25 nM per well and using 4 μl of DharmaFECT 3 reagent (Dharmacon) according to the manufacturer’s instructions. Knockdown efficiency was monitored at the mRNA level by real-time RT-PCR.

*Lipid and Oxysterol Levels Determination*—Levels of 24S-OH and 27-OH in plasma or cerebrospinal fluid (CSF) were assayed by isotope dilution-mass spectrometry as described previously (15). One ml of plasma was required for the assay. In CSF, 10 ng of [2H8]24S-OH and 10 ng of [2H8]27-OH were added to 500 μl of CSF together with 10 μl of butyrylhydroxytoluene (5 mg/ml) and 20 μl of EDTA (10 mg/ml). Under these conditions, the limit of detection in CSF was about 0.2 ng/ml for both oxysterols. Total cholesterol, LDL-cholesterol, and HDL-cholesterol were measured by routine standard enzymatic assay (Modular Analytics; Roche Diagnostics). Determination of the levels of 24S-OH and 27-OH from in vitro experiments were assayed following the same protocol and using 1.5 ml of conditioned media.

*CSF Sample Extraction*—CSF was collected for diagnostic purposes by lumbar puncture in polypropylene tubes, mixed gently to avoid gradient effects, and centrifuged at 2000 × g for 10 min. Aliquots were stored at −80 °C until the biochemical analysis. All individuals gave their informed consent to participate in the study, which was conducted according to the provisions of the Helsinki Declaration.

*Statistical Analysis*—Normality was checked by Shapiro-Wilk test before statistical analysis. Results were analyzed by one-way analysis of variance followed by Tukey’s post hoc test, Student’s t test, or Mann-Whitney’s U test, depending on the number of groups and the parametric or non-parametric conditions. A value of $p < 0.05$ was considered statistically significant.
RESULTS

Cholesterol-enriched Diet Up-regulates the Renin-Angiotensin System in Mouse Brain—To study the in vivo effects of hypercholesterolemia on brain RAS, C57BL/6 mice were fed a HFD containing 21% fat and 0.15% cholesterol (R638, Lactaminne, Sweden) for 9 months. The plasma levels of cholesterol, high-density lipoproteins, and low-density lipoproteins were approximately doubled in HFD-fed animals as compared with controls (supplemental Fig. S1). No significant changes in oxysterol levels were detected in the brains of these animals (data not shown).

Using RT-PCR analysis, we found an up-regulation of ACE expression in the brains of mice fed with a HFD (Fig. 1A), confirming our previously published results using cDNA microarrays (12). As seen in Fig. 1B, AGT protein levels were increased in HFD animals to $\sim130\%$ of those of controls ($p < 0.05$ versus ND, $n = 6$). No changes were found in AngI/II levels. Histograms show data normalized as the ratio to actin levels, and expressed as a percentage of values from ND animals. Data are presented as a mean ± S.E. ND, normal diet.

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pared with controls (Mann-Whitney U = −1.96, n = 6, p < 0.05).

**Up-regulation of RAS by 24S-OH and 27-OH in Rat Primary Neurons**—Although cholesterol itself is unable to pass the BBB, cholesterol homeostasis in the CNS is maintained by an efflux of brain 24S-OH and an influx of blood 27-OH. We next investigated whether these oxysterols modulate RAS in vitro, using rat neuronal primary cultures. We analyzed the effects of either 24S-OH or 27-OH at 3 or 24 h on AGT, ACE, or AT1R expressions.

Treatment with either 24S-OH or 27-OH (1 μM, 24 h) resulted in a significant increase of AGT (n = 6; p < 0.01), ACE (n = 6; p < 0.05), and AT1R (n = 6; p < 0.01) expressions (Fig. 2, A, C, and D) but a decrease of renin expression (n = 6; p < 0.01) (Fig. 2B). No significant effects on AGT, ACE, and AT1R gene expressions were found with 3 h treatments of 1 μM 24S-OH or 27-OH (supplemental Fig. S2A). However, renin expression was found to be decreased with a 3 h treatment of 1 μM 24S-OH (supplemental Fig. S2A).

In view of the effects found on gene expression, we next examined the effects of 24S-OH and 27-OH (1 μM, 24 h) on the levels of RAS proteins. Analysis of cell extract and conditioned media from treated cells revealed that both 24S-OH and 27-OH (1 μM, 24 h) enhanced intracellular (n = 6; p < 0.05; Fig. 2E) and secreted levels of AGT (n = 6; p < 0.01, Fig. 2F). Moreover, as seen in Fig. 2G, treatment of rat primary neurons with either 24S-OH or 27-OH (1 μM, 24 h) increased the phosphorylation of STAT3 at Tyr-705 (n = 9, p < 0.05). This effect was not seen when cells were preincubated for 30 min with the specific AT1R antagonist losartan at 10 (Fig. 2H) or 1 μM concentrations (supplemental Fig. S2B).

The stability of oxysterols (1 μM) was investigated by determining the residual concentrations after 3 or 24 h treatments. After 3 h in contact with the cells, only 3% of 27-OH and 5% of 24S-OH were detected in the media. Moreover, after a 24 h treatment, no measurable concentrations of oxysterols were found in the media. With increased concentrations of oxysterols (10 μM), only 33%, or 35% after 3 h, and 16 or 2% after 24 h, of 27-OH and 24S-OH, respectively, remained. In the extracts of treated cells, the oxysterols levels were ~4% for 27-OH and 18% for 24S-OH. Our findings conclude that both 24S-OH and 27-OH are rapidly integrated and metabolized in the cells.

**Increased Production of AGT in Astrocytes Treated with 24S-OH or 27-OH**—Because astroglia are thought to be the most important source of AGT in the brain (18), we tested the impact of 24S-OH and 27-OH on AGT production in rat primary astrocytes. As shown in Fig. 3A, treatment of astrocytes with 1 μM 24S-OH or 27-OH resulted in a slight but non-significant increase in the cellular levels of AGT (p = 0.22). However, analysis of conditioned media from treated cells revealed a significant enhancement of secreted AGT after 27-OH treatment (p < 0.01) (Fig. 3B). There were no significant effects of 27-OH on renin, ACE, or AT1R gene expressions in astrocytes (Fig. 3, C–E). However, we found a significant down-regulation of renin with 24S-OH (Fig. 3C).

**Oxysterol (24S-OH or 27-OH)-induced Up-regulation of RAS Is Mediated by Liver X Receptors**—Both 24S-OH (19) and 27-OH (20) are endogenous ligands for LXR. To explore if the effects of 24S-OH or 27-OH on RAS are LXR dependent, we used rat primary neuronal cultures and analyzed RAS components in the presence of an agonist or a blocker of LXR. As an agonist, we used 1 μM TO-901317, a potent synthetic LXR agonist that shows no selectivity for either LXRα or LXRβ (21). As a LXR blocker, we used 22(S)-OH (10 μM (22)), an inactive isomer of 22(R)-OH. Cells were pretreated with 22(S)-OH for 3 h before the addition of 24S-OH or 27-OH (1 μM) directly to the medium.

As shown in Fig. 4A, AGT mRNA expression was markedly decreased by TO-901317 (p < 0.01) but increased by 24S-OH and 27-OH (p < 0.01 for both). Pretreatment with 22(S)-OH abolished the AGT up-regulation caused by oxysterols (24S-OH + 22(S)-OH versus 24S-OH, p < 0.01; 27-OH + 22(S)-OH versus 27-OH, p < 0.01). Renin expression was down-regulated by TO-901317 (p < 0.01), 24S-OH (p < 0.01), and 27-OH (p < 0.05) treatments. These effects were counteracted by the blocking of LXR (24S-OH + 22(S)-OH versus 24S-OH, p < 0.01; 27-OH + 22(S)-OH versus 27-OH, p = 0.12) (Fig. 4B).

Treatment with TO-901317 caused a similar increase in ACE expression as 24S-OH and 27-OH (p < 0.01). Again, pretreatment with 22(S)-OH abolished the effects of both oxysterols on ACE expression (24S-OH + 22(S)-OH versus 24S-OH, p < 0.01; 27-OH + 22(S)-OH versus 27-OH, p < 0.01; Fig. 4C). Furthermore, TO-901317 treatment also increased AT1R expression (p < 0.01), as seen by 24S-OH (p < 0.01) and 27-OH (p < 0.05). However, the presence of the LXR blocker did not completely abolish the up-regulation of AT1R induced by either 24S-OH or 27-OH (Fig. 4D).

The decrease in AGT mRNA expression by TO-901317 treatment was somehow surprising. When we analyzed the levels of AGT protein in cell extracts by immunoblotting, treatment with the LXR agonist TO-901317 did not induce significant changes (data not shown; p = 0.99). However, a significant increase in secreted AGT levels were found in the presence of TO-901317 (p < 0.01) as well as with both oxysterols (Fig. 4E. p < 0.01). Moreover, treatment with the LXR blocker 22(S)-OH failed to balance the enhancements of intracellular AGT levels induced by either 27-OH or 24S-OH (data not shown), but was able to inhibit the effects on AGT secretion (Fig. 4E). In addition, TO-901317 also induced similar effects on astrocytes, namely a down-regulation of AGT (p < 0.05) and renin (p < 0.01) mRNA expressions, as well as increased AGT secretion (p < 0.05) (Fig. 4, F and G).

To further demonstrate the key role of LXR on oxysterol-mediated brain RAS up-regulation, we performed LXR knockdown experiments in rat primary neurons. Because LXRβ is widely expressed in the rat fetal brain (23) and at especially high levels in the central nervous system of the adult brain (24), we suppressed LXRβ expression by siRNA. The knockdown efficiency of LXRβ siRNA was relatively high; 2−ΔΔC_{q sistem} = 0.38 ± 0.019 versus 2−ΔΔC_{q sistem} = 1 ± 0.04 (p < 0.01) (Fig. 5A.).

Treatments with 24S-OH and 27-OH preserved this efficiency of LXR knockdown by siRNA (0.41 ± 0.08 and 0.52 ± 0.02, respec-
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The attenuation of LXRβ expression abolished the oxysterol-induced AGT, ACE, and AT1R up-regulations as well as renin down-regulation in rat primary neurons (Fig. 5, B, D, E, and C, respectively). Globally, these results suggest that oxysterols regulate the expression of several members of RAS (AGT, Renin, ACE, and AT1R) by a LXRβ-dependent mechanism.

Brain RAS in Cortical and Hippocampal Areas from Cyp27KO Mice—To further analyze the impact of 27-OH on brain RAS activation, we used Cyp27KO mice. These animals have a deficiency of sterol 27-hydroxylase (Cyp27a1), the initial enzyme in the degradation of side chain steroids and consequently responsible for the synthesis of 27-OH from cholesterol (25). As shown in Fig. 6A, non-significant changes in ACE expression were found in both the cortex and hippocampus of Cyp27KO compared with wild type mice. However, Western blotting analysis revealed a decrease in AGT levels (n = 8, p < 0.05) and JAK/STAT activation (n = 8, p < 0.01) in Cyp27KO to ~80% of levels seen in wild type (WT) mice for both brain regions (Fig. 6B).

Markers of RAS Activation in the CSF of SPG5 Patients Are Associated with High Levels of 27-OH—To additionally test the hypothesis that increased 27-OH levels result in an overactivation of brain RAS, we next analyzed RAS members in SPG5 patients. These patients have a mutation in the gene coding for the oxysterol 7α-hydroxylase (Cyp7b1) resulting in a recessively inherited “pure” hereditary spastic paresis (26). One of the consequences of such a mutation is the accumulation of 27-OH due to decreased enzyme activity (27). SPG5 is a rare disease in which patients have symptoms of neurodegenerative...
processes such as progressive paraplegia, cerebellar ataxia, optic atrophy, and sometimes mental retardation (26). As reported in our previous article (27), plasma levels of 27-OH were 6–9-fold higher compared with controls in these patients (Table 1). In the CSF, 27-OH levels were also increased by 30–50-fold compared with controls. However, plasma and CSF levels of 24S-OH were normal because this oxysterol is not a substrate of Cyp7b1. Additionally, no changes were found in total cholesterol levels in the plasma. Immunoblotting analysis of CSF revealed that SPG5 patients have higher levels of both

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![Graphs and images showing relative expression of AGT, Renin, and AT1R with various treatments.](image-url)
AGT and AngI/II in the CSF, as compared with controls (Z = −1.96, n = 6, p < 0.05; for both measurements; Fig. 7).

**DISCUSSION**

An increasing number of studies support a relationship between hypercholesterolemia, hypertension, and neurodegeneration (28). The results presented here are consistent with the contention that the cholesterol metabolites, 24S-OH and 27-OH, are regulators of the brain RAS by a LXR-dependent mechanism.

In an attempt to clarify as to whether this finding really refers to a potential role for the metabolism of cholesterol in the modulation of RAS, we have studied the components of this system in different models including: 1) an animal model of hypercholesterolemia induced by cholesterol-enriched diet; 2) different cell cultures treated with 24S-OH and 27-OH; 3) an animal model deficient of sterol 27-hydroxylase (Cyp27a1) and therefore do not synthesize 27-OH; and 4) patients with high levels of 27-OH due to a mutation in the gene coding for the oxysterol 7α-hydroxylase (Cyp7b1).

Several studies have shown that HFD induces cognitive deficits in mice (29, 30). ApoE knock-out mice fed with HFD showed increased levels of 27-OH but unchanged levels of 24S-OH in the brain (31). We recently showed that HFD induced changes in the expression of several genes in mice brain (12). On the other hand, recent papers reported that transgenic mice expressing human RENIN and AGT genes (hRN/hAGT-Tg) have impaired cognition, increased oxidative stress, and ischemic damage in the brain mediated by over-stimulation of AT1R (32, 33).

In the present study, we demonstrated that a cholesterol-enriched diet caused a significant up-regulation of ACE as well as increased levels of AGT in mice brains. As could have been expected (16, 17) the increased AGT levels were associated with increased activation of the JAK/STAT pathway, reflecting an activation of AT1R. Because cholesterol does not pass the BBB but cholesterol metabolites 27-OH and 24S-OH do, we next tested the effects of these oxysterols on RAS in vitro. We confirmed that both 24S-OH and 27-OH (1 μM, 24 h) are able to up-regulate ACE and AGT expression in rat primary cultures. In addition, both oxysterols induced AT1R up-regulation in neurons, and as a consequence JAK/STAT activation with high levels of Tyr-705-phosphorylated STAT3. These results were confirmed by blocking AT1R using losartan; where the JAK/STAT activation was abolished.

A potential role of LXRs as key players in RAS and blood pressure regulation has been previously reported in the periph-
eral vascular system (34). Because both 24S-OH (19) and 27-OH (20) are endogenous ligands of LXR, we next determined whether the above effects of the oxysterols could be mediated by LXR. First, AGT expression was affected by LXR stimulation with TO-901317. This LXR agonist markedly reduced AGT expression in both neuronal and astrocyte cultures. In the media of these cultures, however, high levels of AGT protein were found when the cells were exposed to LXR agonists. This high accumulation of AGT in the medium could be responsible for a compensatory decrease in AGT expression by the cells. A similar negative feedback mechanism has been seen in the synthesis and secretion of apolipoprotein E (apoE) (35), and it is noteworthy to mention that the synthesis of apoE is also controlled by LXR (36). Additionally, TO-901317 treatment reproduced the effect of oxysterols on renin and AT1R expressions.

Second, LXR silencing by the LXR blocker (22(S)-OH) abolished the oxysterol-induced ACE up-regulation. Despite the fact that AngII up-regulates AT1R expression (37), we failed to demonstrate an inhibitory effect with LXR blockers on AT1R expression. According to a previous study, RAS inhibition is compensated by an increase in Renin activity, which stimulates the conversion of AngI and ultimately AngII (38). In vitro data have demonstrated a conversion of AngI to AngII via ACE-independent pathways, including chymase, cathepsin D, and chymotrypsin-like angiotensin-generating enzyme (39). Because LXR activation led to a decreased expression of renin mRNA, this compensatory mechanism may explain our finding that 22(S)-OH abolished the oxysterol-induced ACE up-regulation but not the AT1R expression.

The central role of LXR on brain RAS up-regulation induced by oxysterols was further confirmed by siRNA experiments in rat primary cultures. Considering that the β isoform is predominantly expressed in rat fetal brain (23) and in the central nervous system (24), we decided to knockdown LXRβ and explore the effects of 24S-OH and 27-OH on brain RAS. We demonstrated that changes in AGT, renin, and AT1R expressions previously found with 24S-OH and 27-OH were abolished by the suppression of LXRβ expression. However, LXRβ knockdown did not have a major effect on the oxysterol-mediated up-reg-

### TABLE 1

| Patient | Controls | Case 1 | Case 2 | Case 3 |
|---------|----------|--------|--------|--------|
| Plasma 27-OH (ng/ml) | 89–243 | 1213 | 1316 | 1046 |
| Csf 27-OH (ng/ml) | 0.5–0.8 | 14 | 14 | 24 |
| Plasma 24S-OH (ng/ml) | 30–127 | 52 | 62 | 66 |
| Csf 24S-OH (ng/ml) | 0.8–2.4 | <0.5 | <0.5 | 1 |
| Plasma cholesterol (mmol/liter) | 3.5–8 | 4.3 | 4.5 | 4.1 |

**FIGURE 6. Brain RAS in Cyp27KO mice.** A, ACE mRNA was analyzed in two brain regions from WT and Cyp27KO mice: cortex and hippocampus. No significant changes were found in ACE expression between both groups. Data are presented as mean ± S.E. B, cortical and hippocampal samples from WT and Cyp27KO mice were immunoblotted with AGT and p-STAT3(Tyr-705) antibodies. Histograms show data normalized as the ratio to actin levels, and expressed as a percentage of values from WT animals. Data are presented as mean ± S.E. (*, p < 0.05; **, p < 0.01). WT, wild type; Cyp27KO, sterol 27-hydroxylase (Cyp27a1)-deficient mice.
ulation of ACE expression. Our findings with the LXR antagonist and knockdown suggest that ACE expression is not regulated by a LXRα-dependent mechanism. A possible involvement of the LXRβ isoform of LXR cannot be completely ruled out because up-regulation of ACE was abolished with the LXR blocker, but not with LXRβ knockdown. A low proportion of LXRβ is expressed in primary neurons, and LXRβ knockdown did not affect LXRβ expression (data not shown).

In addition, it is evident that LXRs are not exclusive mediators of oxysterol-induced effects. Other nuclear receptors have been described to be activated by oxysterols, including pregnane X receptor (40) and retinoic acid receptor-related orphan receptors (41), which are expressed in multiple tissues including the brain. It has been suggested that oxysterols could bind and activate LXR at physiological concentrations, while acting through the pregnane X receptor at high pharmacological doses (42). The relative amount of pregnane X receptor is lower than that of LXRβ in the brain, whereas in the liver they are in equal amounts (43). The putative contribution of pregnane X receptor and retinoic acid receptor-related orphan receptors in the effect seen by oxysterols on the brain RAS remains to be investigated.

The effects observed in vitro after exposure to oxysterols are somewhat similar to the effects of hypercholesterolemia in mice brains. Thus, it seems possible that the increased RAS activity in the brain, induced by hypercholesterolemia, may be mediated by the BBB-permeable cholesterol metabolite 27-OH. Hypercholesterolemia is likely to lead to elevated levels of 27-OH in the circulation and a higher influx to the brain. Treatment of apoE knock-out mice with a high cholesterol diet have increased levels of 27-OH in the brain (31). However, in our study, we did not find significantly elevated brain levels of 27-OH in mice treated with a cholesterol-enriched diet, which seems to be in contradiction with this hypothesis.

To shed light on this apparent inconsistency, we used two approaches. First, we analyzed the metabolism of oxysterols in vitro, determining the residual concentrations of 27-OH and 24S-OH in the media after treatment of cells for either 3 or 24 h. Two concentrations were used (1 or 10 μM), and our results demonstrate that both oxysterols were rapidly incorporated and metabolized by primary neurons.

Second, we used another animal model (Cyp27KO) in which no 27-OH is synthesized. We showed that AGT levels and JAK/STAT activation were reduced in cortical and hippocampal areas from these mice. However, no changes in ACE activity were found.

In general, the results that we obtained in in vitro models were larger than those obtained in in vivo models. It should be emphasized, however, that the brain metabolism of 27-OH is very effective and as a consequence the levels would be very low in this organ. Thus, it seems likely that the flux of 27-OH into the brain is more important than the levels measured postmortem.

Also, we can envisage the existence of compensatory mechanisms to counteract the overactivations of LXRs and RAS in the brain. These compensatory mechanisms could be compromised with age, or under neuropathological conditions.

We further explored the possibility that increased levels of 27-OH in the blood have consequences for the brain RAS in humans. For that, we analyzed the CSF of a subgroup of patients with hereditary spastic paresis, SPG5. These patients carry mutation(s) in the gene coding for Cyp7b1 (26), and have markedly increased levels of 27-OH but normal cholesterol levels in the plasma and CSF (27). In agreement with our hypothesis, we found that CSF from SPG5 patients had significantly increased levels of AGT and AngII. The results of the present study suggest that 27-OH may operate as a link between the neurodegenerative effects caused by high blood cholesterol levels and hypertension.
Patients with advanced AD have increased levels of 27-OH in the brain (44), and we have discussed the possibility that there is a relationship between 27-OH and dementia (10). We have recently published that AGT and ACE are increased in the CSF of patients with mild cognitive impairment and AD. Additionally, ACE activity in the CSF was positively correlated with both plasma and CSF levels of 27-OH (45). Accumulating data support an association between hypertension with cognitive functions (46), although insufficient evidence showed that antihypertensive drugs can really prevent cognitive decline or dementia (47). Animal studies have shown that ACE inhibitors enhance cognitive performance (48), and improve learning and retention of memory (49). On the other hand, overstimulation of AT1R leads to cognitive deficits and ischemic damage in mice (32, 33), as well as hypertension, vascular brain damage, and insulin resistance in humans (50). Thus, it is tempting to suggest that a hypercholesterolemia-mediated overactivation of brain RAS, via the BBB permeable 27-OH, may contribute to neurodegenerative processes including memory impairment.

It should be mentioned that the flux of 27-OH from the blood into the brain might accentuate neurodegeneration by mechanisms other than the one reported here. We have recently shown that 27-OH reduces the expression of the activity-regulated cytoskeleton-associated protein (Arc), an important factor for synaptic potentiation and consolidation of memory (12). 27-OH has also been shown to antagonize the preventive effects of 24S-OH in the generation of Aβ (10), and increases Aβ production in neuronal preparations from adult rabbit brain (51).

In summary, within the present study we found that under in vitro conditions both 27-OH and 24S-OH activate brain RAS, by a LXR-mediated mechanism (Fig. 8). HFD diet resulted in an overactivation, whereas depletion of 27-OH resulted in down-regulation of brain RAS. To further support the hypothesis that 27-OH is responsible for a mechanism by which high blood cholesterol could contribute to neurodegenerative disorders, we showed that SPG5 patients have high levels of AGT and AngII in the CSF, reflecting an increased RAS activity in the brain.

Overall, our results are consistent with: 1) one of the biological functions of 24S-OH and 27-OH is to modulate RAS activity; 2) stimulation of LXR enhances RAS activity in brain cells; and 3) high blood cholesterol could contribute to neurodegeneration through a 27-OH-induced overactivation of RAS in the brain.

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