Cholestenoic acids regulate motor neuron survival via liver X receptors

Spyridon Theofilopoulos,1 William J. Griffiths,2 Peter J. Crick,2 Shanzheng Yang,1 Anna Meljon,2 Michael Ogundare,2 Satish Srinivas Kitambi,1 Andrew Lockhart,3 Karin Tuschi,4 Peter T. Clayton,4 Andrew A. Morris,5 Adelaida Martinez,6 M. Ashwin Reddy,7 Andrea Martinuzzi,8 Maria T. Bassi,9 Akira Honda,10 Tatsuki Mizuochi,11 Akihiko Kimura,11 Rebecca Schüle,15,16 Ludger Schöls,15,16 Andreas W. Sailer,17 Jens Kuhle,18 Matthew J. Fraidakis,19 Jan-Åke Gustafsson,20,21 Knut R. Steffensen,22 Ingemar Björkhem,2 Pátrik Ernfors,1 Jan Sjövall,1 Ernest Arenas,8 and Yuqin Wang2

Introduction

The vertebrate CNS is composed of a wide variety of neurons that are generated following tightly regulated developmental programs. Characterization of the function and specificity of molecules working on distinct neuronal populations is thus essential in order to enhance our understanding of how such complexity is achieved in the developing brain and how it is maintained in the adult brain. One means of developmental and adult regulation is via nuclear receptors. Examples of nuclear receptors expressed in embryonic and adult brain having both a developmental role (1) and a function in the adult brain (2) are the liver X receptors (LXRs), which are activated by oxysterols (3, 4). Analysis of double-knockout Lxra−/−Lxrb−/− mice revealed that LXRs are required for neurogenesis during ventral midbrain (VM) development (1). Moreover, adult male Lxrb−/− show progressive accumulation of lipids in the brain and loss of spinal cord motor neurons, suggestive of a neuroprotective role of LXRs and their ligands on adult motor neurons (5). Similarly, the number of islet-1−oculomotor neurons is lower in the developing midbrain of Lxra−/−Lxrb−/− mice, indicating a role of LXRs not only in the maintenance of

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adult motor neurons, but also in their development (1). In agreement with these findings, enzymes involved in the synthesis of cholesterol and oxysterols, such as 2,3-oxidoredoxolane-lanosterol cyclase, are localized in islet-1 oculomotor neurons in the mouse VM at E11.5 (1). In addition to the above, we recently found that oxysterols and other endogenous brain LXR ligands are sufficient to regulate neurogenesis in the developing VM (1, 6). While endogenous brain LXR ligands have been identified and found to regulate the development of midbrain dopamine neurons and red nucleus neurons (6), to date, no endogenous ligand capable of regulating the survival of motor neurons in vivo has been identified. In a recent study, we found that cholesterol metabolites that had the capacity to activate LXRs could be identified in human cerebral spinal fluid (CSF) (7). In order to identify novel LXR ligands that regulate motor neuron function, we delved deeper into the human CSF sterolome and examined plasma of patients with 2 different human diseases associated with upper motor neuron degeneration, hereditary spastic paraparesis type 5 (SPG5) and cerebrotendinous xanthomatosis (CTX). These diseases result from mutations in the cytochrome P450 (CYP) genes CYP7B1 and CYP27A1, respectively (8–17). The enzymes coded by these genes are responsible for 7α-hydroxylation of oxysterols and (25R),26-hydroxylation of sterols, respectively, reactions that generate further oxysterols and ultimately cholestenoic acids (Figure 1A and ref. 18). Note that we have adopted the sterol nomenclature recommended by the lipid maps consortium: 26-hydroxycholesterol (26-HC) refers to cholest-5-ene-3β,26-diol; similarly, carboxylic acids that introduce 25R stereochemistry to the side-chain are at C-26 (19). We found that specific cholestenoic acids with a 3β-hydroxy-5-ene, but not a 3-oxo-4-ene, structure activated LXRa and LXRβ in neuronal cells, increased expression of islet-1, a transcription factor required for the development of motor neurons (20–22), and promoted the survival of islet-1 oculomotor neurons. Moreover, these effects were abolished by knockdown or knockout of the LXR-encoding genes in zebrafish or in rodent models, respectively. In addition, we showed that patients with CTX and SPG5 are unable to synthesize normal amounts of the LXR ligand 3β,7α-dihydroxycholester-5-en-26-oic acid (3β,7α-dihCA), a cholestenoic acid that we found to promote neuronal survival. This is of interest in relation to the fact that patients with SPG5 present with motor neuron degeneration and spastic paraplegia. Patients with CTX may sometimes also present with spasticity, possibly due to upper motor neuron degeneration. Our findings may have important implications for neurological diseases leading to motor neuron degeneration, since LXR ligands, as well as inhibitors of specific biosynthetic enzymes in the cholestenoic acid biosynthetic and metabolic pathways, may be useful pharmaceuticals for the treatment of motor neuron disorders.

**Results**

**Specific cholestenoic acids are abundant in human CSF.** We have previously shown that cholestenoic acids are abundant in human plasma (23, 24) and that human CSF also contains specific cholestenoic acids (7). Surprisingly, in CSF, the levels of cholestenoic acids were higher than those of oxysterols. In this study, we delved deeper into the CSF sterolome, particularly into the cholestenoic acid portion, using liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) analysis. We hereby report the exact identity of 16 oxysterols and downstream metabolites, including cholestenoic acids, found in human CSF (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI68506DS1). The most abundant of these metabolites (19.48–0.40 ng/ml; Supplemental Figure 1) were 7α-hydroxy-3-oxocholest-4-en-26-oic acid (7αH,3O-CA), 3β-hydroxycholesterol-5-en-26-oic acid (3β-HCA), and 2 newly identified metabolites in CSF, 3β,7α-dihHCA and 3β,7β-dihydroxycholesterol-5-en-26-oic acid (3β,7β-dihHCA). Precursors of these acids, including 26-HC and newly identified 7α,26-dihydroxycholesterol (7α,26-dH; cholesterol-5-ene-3β,7α,26-triol) and 7α,26-dihydroxycholesterol-4-en-3-one (7α,26-dHO), were also found, but at lower levels (0.15–0.03 ng/ml). Our results thus identified 4 novel oxysterol metabolites in human CSF that were downstream of 26-HC (Figure 1A). 26-HC is metabolized via 7α,26-dihH and 7α,26-dHO, or via 3βHCA and 3β,7α-dihHCA, to 7αH,3O-CA. While 26-HC can cross the blood–brain barrier (BBB) and enter the brain from the circulation (25), 7αH,3O-CA traverses the BBB and is exported from the brain (26). Very low levels of 24S-hydroxycholesterol (24S-H; cholesterol-5-ene-3β,24S-diol), 25-hydroxycholesterol (25-H; cholesterol-5-ene-3β,25-diol), and newly identified 7α,25-dihydroxycholesterol (7α,25-dH; cholesterol-5-ene-3β,7α,25-triol) and 7α,25-dihydroxycholesterol-4-en-3-one (7α,25-dHO) were also found in CSF (0.08–0.03 ng/ml).

**Reduced levels of 7α-hydroxylated cholestenoic acids in CSF and plasma/serum of human patients with SPG5.** SPG5 presents with upper motor neuron signs and results from mutations in CYP7B1, encoding the oxysterol 7α-hydroxylase responsible for 7α-hydroxylation of side-chain oxidized sterols that is required for extrahepatic synthesis of 7αH,3O-CA and its precursor, 3β,7α-dihHCA (Figure 1A and ref. 18). In order to examine the pathogenic role of such mutations, we sought to identify alterations in oxysterol and cholestenoic acid profiles in CSF and plasma from these patients and then examine the biological activities of the altered metabolites. We first studied the CSF from 3 patients with SPG5 (see Supplemental Table 3 for patient clinical data). Elevated levels of the CYP7B1 substrates 25-HC (P < 0.10), 26-HC (P < 0.07), and 3β-HCA (P < 0.02), as well as reduced levels of its products, 3β,7α-dihHCA (P < 0.03) and 7αH,3O-CA (P < 0.001), were found compared with 18 individual control subjects; similar results were found when the SPG5 patients were compared with 2 healthy carrier heterozygotes with a single mutation in CYP7B1 (Figure 1, F–I, and Supplemental Table 1). When plasma was analyzed from 9 SPG5 patients (8, 10, 14), significantly elevated 25-HC (P < 0.03), 26-HC (P < 0.001), and 3β-HCA (P < 0.02) and reduced 3β,7α-dihHCA (P < 0.001) and 7αH,3O-CA (P < 0.02) were found compared with control subjects (Figure 1, B–E, and Supplemental Table 2). Similar differences were found between patient samples and 3 healthy carriers. This indicates that for these metabolites, plasma represents a good surrogate for CSF. However, while 3β,7α-dihHCA and 7αH,3O-CA in the CNS is normally derived from 26-HC, that found in the circulation can be derived via either the 26-HC or the 7α-hydroxycholesterol (7α-H; cholesterol-5-ene-3β,7α-diol) pathway (acidic and neutral, respectively) of bile acid biosynthesis (18). Thus, in SPG5 patients (CYP7B1 mutation), the liver-specific 7α-hydroxylase CYP7A1 (neutral pathway) accounts for the residual content of 3β,7α-dihHCA and 7αH,3O-CA found in the

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circulation. We previously investigated the plasma oxysterol and cholestenoic acid profile of 3 infants with mutations in CYP7B1 (Supplemental Table 2) resulting in oxysterol 7α-hydroxylase deficiency (O7AHD) and neonatal liver disease (27–30), as well as SPG5 in adults (31). The first identification of CYP7B1 mutations were found in a child with severe cholestasis (32), defining a new inborn error of bile acid biosynthesis. As expected by the absence of functional CYP7B1 in these patients, we found very low plasma levels of 3β,7α-diHCA (P < 0.001; Figure 1D and Supplemental Table 2), as described above for SPG5. These patients also had considerably elevated plasma levels of 24S-HC, 25-HC, and 26-HC and high levels of hepatotoxic 3β-hydroxychol-5-en-24-oic acid (3β-HΔ5-BA) compared with SPG5 patients and controls. These findings suggest that additional factors, including increased levels of toxic 3β-hydroxy-5-ene acids, may contribute to the progressive liver disease in these patients at an early age.

CTX is a second human disease that may present with signs of motor neuron loss. It is characterized by mutations in CYP27A1.

We found that the plasma of patients with CTX was essentially devoid of 26-HC and the downstream cholestenoic acids (Figure 1, B–E, and Supplemental Table 2). As reported previously (33), elevated levels of 7α-HΔ3-ene-3-one (7α-HCO) and/or 7α,12α-dihydroxycholesterol (7α,12α-diHCh; cholest-5-ene-3β,7α,12α-triol) plus 7α,12α-dihydroxycholesterol-4-en-3-one (7α,12α-diHCO) were observed (Supplemental Table 2). The absence of cholestenoic acids in plasma indicates an inability to biosynthesize C27 acids in extrahepatic steroidogenic tissue, including the CNS.
In summary, our data showed specific changes in $7\alpha$-hydroxylated cholestenoic acids in plasma of CTX and SPG5 patients; we thus decided to examine their effect on neural function.

Specific cholestenoic acids are ligands to LXR$\alpha$ and LXR$\beta$ in neural cells. In order to gain insights into the mechanism by which alterations in cholesterol metabolism causes neurological disease, we studied whether any of the cholestenoic acids present at high levels in control human CSF and deregulated in CSF or plasma of SPG5 or CTX patients work as LXR ligands. We thus focused on $26$-HC, $3\beta$-HCA, $3\beta,7\alpha$-diHCA, its isomer $3\beta,7\beta$-diHCA, and $7\alpha$H,3O-CA (Figure 1A) and tested their capacity to activate LXR$\alpha$ and LXR$\beta$ in neural cells. We previously found that $3\beta$-HCA activates LXR$\beta$ (7) and here observed that $3\beta,7\alpha$-diHCA, its isomer $3\beta,7\beta$-diHCA, and the necessary intermediate for the interconversion of the isomers, $3\beta$-hydroxy-7-oxocholest-5-en-26-oic acid ($3\beta$H,7O-CA), had the ability to activate both LXRs and therefore act as LXR ligands in neural cells (Figure 2A and Supplemental Figure 2, A and B). Values in A–C are fold activation over the basal LXRE or FXRE luciferase activity (set to 1) ($n = 3$). *$P < 0.05$, **$P < 0.01$ vs. vehicle or as indicated by brackets, Mann-Whitney test. (D) TR-FRET LXR$\beta$ coactivator assay, used to determine the binding affinity of cholestenoic acids as well as the known LXR ligands GW3965 and 24,25-EC toward the LXR$\beta$-LBD ($n = 3$). The 520:495 TR-FRET ratio was determined as described in Methods. *$P < 0.05$ vs. vehicle for $\geq 10 \mu M$ $3\beta,7\alpha$-diHCA, $\geq 10 \mu M$ $3\beta,7\beta$-diHCA, $\geq 5 \mu M$ $3\beta$-HCA, $\geq 24.25\mu M$, and $\geq 5 \mu M$ GW3965, Mann-Whitney test. (E) $3\beta,7\alpha$-diHCA, $3\beta,7\beta$-diHCA and $3\beta$-HCA induced significant increases in $Abca1$, $Abcg1$, and $Srebf1$ in SN4741 cells ($n = 3$). *$P < 0.05$, **$P < 0.01$ vs. vehicle, Mann-Whitney test.
had the lowest 50% effective concentration (EC_{50}), followed by 3β,7α-diHCA and 3β-HCA. In addition, we confirmed in our system the capacity of 24S-HC and 25-HC to activate LXR and observed that 26-HC had no significant effect. We also tested the activation capacity of 7αH,3O-CA, its 7β-isomer, 7β-hydroxy-3-oxocholesterol-4-en-26-oic acid (7βH,3O-CA), and the precursors 7α,26-diHCA and 7α,26-diHC0, none of which showed significant activity (Figure 2A and Supplemental Figure 2A). Moreover, in order to examine whether the identified acidic ligands exert their effect by binding to LXR, we used the LXR antagonist geranylgeranyl pyrophosphate (GGPP; ref. 34), which blocked their activity (Figure 2C), indicating that the acids are indeed LXR ligands. The activity of the known LXR ligands 22R-HC and 25-HC was similarly blocked. In order to confirm that the cholestenoic acids are indeed agonists to LXR, we tested the LXRβ activational capacity of 3β,7α-diHCA, 3β,7β-diHCA, or 3β-HCA in combination with 22R-HC. No additive effect was observed (Supplemental Figure 3A), which indicates that these cholestenoic acids work, as does 22R-HC, via LXR. To further examine the specificity of the cholestenoic acids described above, farnesoid X receptor (FXR) luciferase reporter assays were performed (Figure 2B). As previously described (35), chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) activated FXR. Interestingly, 3βH,3α-LH-BA (36), a cholesterol metabolite identified in plasma (Supplemental Table 2), also activated FXR, but was less potent than CDCA. However, none of the other compounds tested activated the FXR luciferase reporter or modulated the activity of CDCA in neural cells (Figure 2B and Supplemental Figure 3B). Thus, the cholestenoic acids do not exert their effect via modulating the activity of the FXR nuclear receptor or its ligands. Similarly, cholestenoic acids did not activate luciferase reporter assays under the control of a DR5 element (activated by Nur-related factor 1/retinoid X receptor [NURR1/RXR] heterodimers), whereas 9-cis-retinolic acid (9-cis-RA) activated this reporter (Supplemental Figure 2D). Furthermore, while lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid) activated a vitamin D receptor (VDR) luciferase reporter, none of the cholestenoic acids tested showed any significant effect on VDR activation in neural cells (Supplemental Figure 2C). Thus, our results indicate that 3β-HCA (7), 3β,7α-diHCA, and 3β,7β-diHCA are specific LXR ligands in neural cells.

**Specific cholestenoic acids bind and activate LXR.** In order to show that specific cholestenoic acids directly interact with LXR, we performed a binding and activation fluorescence resonance energy transfer (FRET) assay, in which ligand binding to the ligand binding domain (LBD) of LXRβ (referred to herein as LXRβ-LBD) recruits a fluorescent coactivator. 3β,7α-diHCA induced FRET, in a dose-dependent manner, to a greater extent than the other acids, but to a lesser extent than the known LXR ligands GW3965 and 24S,25-epoxycholesterol (24S,25-EC; 3β-hydroxycholesterol-5-en-24S,25-epoxide) (Figure 2D). The respective efficacy (GW3965-normalized) and EC_{50} values were 1 and 4.04 μM for GW3965, 0.79 and 4.94 μM for 24S,25-EC, 0.41 and 4.16 μM for 3β,7α-diHCA, 0.25 and 4.51 μM for 3β,7β-diHCA, and 0.27 and 3.83 μM for 3β-HCA. To provide further evidence that cholestenoic acids are LXR ligands in neural cells, we treated neural cells with the individual acids for 3 hours and measured Abca1, Abcg1, and Srebf1 transcript levels. 3β,7α-diHCA increased transcripts levels to a similar degree as 22R-HC, while 3β,7β-diHCA and 3β-HCA induced transcription, but to a lesser extent (Figure 2E). These results provide further proof that 3β-HCA, 3β,7α-diHCA, and 3β,7β-diHCA are specific LXR ligands in neural cells.

3β,7α-diHCA and 3βH,7O-CA increase islet-1 expression in islet-1-GFP zebrafish embryos. Having established that cholestenoic acids that are altered in CTX or SPG5 can activate LXRs in vitro, we next sought to identify their effect in vivo. In particular, we focused on the expression of islet-1, a transcription factor expressed in all postmitotic motor neurons (20, 37) and required for multiple aspects of motor neuron development, including motor neuron specification, motor column formation, axonal growth, and maintenance of spinal motor neuron identity (38). We used transgenic zebrafish embryos expressing GFP driven by the isl1 gene promoter/enhancer sequences (Tg(isl1:GFP)) (39, 40) to screen for biologically active compounds in vivo. Previous studies have indicated that islet-1 protein is required for the formation of zebrafish primary motor neurons and is conserved throughout vertebrate evolution (21). Treatment of Tg(isl1:GFP) embryos with the weak LXR ligands 3β-HCA and 3β,7β-diHCA, as well as CDCA, the most potent FXR ligand, had a deleterious toxic effect that impaired survival and precluded further in vivo analysis. Interestingly, we found that 2 LXR agonists, 3β,7α-diHCA and 3βH,7O-CA, increased islet-1-GFP expression, but had no significant effect on the number of islet-1 cells, in the different cranial nerves examined (loci III, IV, V, VII, and X; Figure 3, A and B). These effects were specific, as 7αH,3O-CA, which is not an LXR ligand, failed to regulate islet-1-GFP expression (Figure 3B). Notably, the increase in islet-1-GFP expression observed in response to the specific acidic compounds was evident in all cranial nerves studied (Figure 3B). Importantly, the increased islet-1-GFP expression in response to 3β,7α-diHCA and 3βH,7O-CA was also accompanied by an increased level of isl1 mRNA, as assessed by quantitative PCR (Figure 3C). To further verify our results, we examined whether these cholestenoic acids regulated the in vivo expression of endogenous LXR target genes, such as abca1. Our results showed enhanced expression of abca1 by both 3β,7α-diHCA and 3βH,7O-CA, but not by 7αH,3O-CA (Figure 3D). Finally, in order to determine whether these effects are actually mediated by LXRs, we performed krox morpholino (MO) injections in Tg(isl1:GFP) zebrafish. Interestingly, we found that krox MO injections abolished the in vivo increase in islet-1-GFP levels by 3β,7α-diHCA and 3βH,7O-CA, compared with control scrambled MO (Supplemental Figure 4). Thus, our data showed that cholestenoic acids are capable of activating endogenous LXR target genes in vivo and regulate the in vivo expression of islet-1 in brain motor neurons via LXR.

3β,7α-diHCA and 3βH,7O-CA increase, whereas 3β,7β-diHCA and 3β-HCA decrease, the number of rodent oculomotor neurons in vitro. In order to determine the functional relevance of our findings in mammalian cells, we examined whether any of the cholestenoic acids present in human CSF and implicated in motor neuron loss are also capable of regulating islet-1 expression in mouse primary brain progenitor cultures. We first performed dose-response analysis of the cholestenoic acids of interest, using a wide range of concentrations; whereas 3β,7β-diHCA and 3β-HCA caused loss of islet-1 cells in the cultures, equimolar concentra-
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The number of oculomotor neurons peaked after 2 days in culture (Supplemental Figure 3B), indicating that they are bona fide oculomotor neurons. Time-course analysis confirmed the stimulatory effect of 3β,7α-diHCA and 3βH,70-CA, as well as the toxic effect of 3β,7β-diHCA and 3β-HCA, over a range of incubation times (Supplemental Figure 5A). The number of oculomotor neurons peaked after 2 days in culture and leveled thereafter. These results confirmed in rodent cells the dual effects of cholestenoic acids first observed in zebrafish, where 3β,7β-diHCA and 3β-HCA caused death, whereas 3β,7α-diHCA and 3βH,70-CA were specific, as they did not affect the number of other midbrain neurons, such as tyrosine hydroxylase–positive (TH+) dopamine neurons, GABAergic neurons, or red nucleus neurons in the cultures (data not shown). In order to examine whether the effects of 3β,7α-diHCA and 3βH,70-CA were specifically mediated by LXR receptors in the rodent brain, we performed progenitor brain cultures from Lxra−/−Lxrb−/− mice. Remarkably, the effects of 3β,7α-diHCA and 3βH,70-CA (at their most efficient concentration) on islet-1+ neurons were eliminated (Figure 4C), confirming that they regulate the number of islet-1+ cells in the rodent brain through LXRs. Moreover, the effects of these acids were blocked by GGPP (Figure 4D), further suggesting that the observed effects were mediated by LXR receptors.

Interestingly, 26-HC, a precursor of cholestenoic acids in the acidic pathway of bile acid biosynthesis (18) that had no LXR activation effect in luciferase assays in neural cells (Figure 2 and ref. 6), also exhibited no effect on the number of islet-1+ cells (data not shown). Furthermore, the effects of 3β,7α-diHCA, 3β,7β-diHCA, and 3β-HCA on islet-1+ cells were not altered by the known LXR ligand 22R-HC (Supplemental Figure 3C), which does not show an effect toward islet-1+ cells (6). These findings are very exciting, as none of the previously known endogenous brain LXR ligands regulates the function of motor neurons. We recently reported that brain endogenous LXR ligands selectively regulate neurogenesis and/or survival of other cell populations in the developing midbrain (6). We thus sought to examine the mechanism by which 3β,7α-diHCA and 3βH,70-CA increase the number of islet-1+ cells and studied their role in neurogenesis, proliferation, and motor neuron survival in the developing brain.

3β,7α-diHCA promotes rodent oculomotor neuron survival in vitro, whereas 3β,7β-diHCA and 3β-HCA are toxic. Neurogenesis was examined in BrdU pulse-chase experiments, in which neuronal progenitors in primary cultures are labeled with a pulse of BrdU at the beginning of the experiment and then are examined for their differentiation into motor neurons, as assessed by the acquisition of

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Figure 4. Specific cholestenoic acids increase the number of islet-1+ oculomotor neurons, promote neuronal survival, or are toxic in mouse E11.5 brain primary cultures. (A) Dose-response curves for quantification of islet-1+ cells in mouse E11.5 brain primary cultures from WT embryos treated with the indicated compounds. *P < 0.05 vs. vehicle for ≥5 μM 3β,7α-diHCA, ≥10 μM 3βH,7O-CA, ≥2 μM 3β,7β-diHCA, and ≥2 μM 3β-HCA, Mann-Whitney test. (B) Representative islet-1– and NKX6.1-stained cell nuclei treated with 3β,7α-diHCA (10 μM), 3βH,7O-CA (10 μM), 3β,7β-diHCA (2 μM), or 3β-HCA (2 μM). (C) Quantification of islet-1+ cells in primary cultures from WT or Lxra–/–Lxrb–/– embryos treated with 3β,7α-diHCA or 3βH,7O-CA (10 μM). *P < 0.05 vs. vehicle, Mann-Whitney test. (D) Effect of 10 μM GGPP on treatment in the WT group. *P < 0.05 vs. vehicle or as indicated by brackets, Mann-Whitney test. All data are mean ± SEM (n = 3). Scale bars: 20 μm.
of islet-1 expression, a marker for motor neuron fate (41). Surprisingly, none of the cholestenolic acids studied affected the number of double-positive BrdU/islet-1+ cells (Supplemental Figure 6A), which indicates that they do not promote motor neuron neurogenesis. Similarly, none of the cholestenolic acids affected the total number of BrdU+ cells in the cultures (Supplemental Figure 6B), which suggests that they do not modulate proliferation. Finally, we tested whether cholestenolic acids regulate neuronal survival, as assessed by active caspase-3 staining to detect the number of cells undergoing apoptosis in the cultures. Interestingly, treatment with 3β,7α-
diHCA over a wide range of concentrations decreased the number of active caspase-3 cells, whereas βH,7O-CA had no effect (Figure 4, E and F). Since proliferation, neurogenesis, and cell death were not affected by βH,7O-CA, we attribute the increase in islet-1 cells to an increase in differentiation, given the known role of islet-1 in this process (20). Cholestenoic acids that reduced the number of islet-1 cells were also examined for their capacity to induce cell death in oculomotor neuron cultures. Interestingly, low concentrations of either βH,7β-diHCA or βH-CA increased the number of active caspase-3 cells (Figure 4, E and F), with toxicity starting at 1 μM. No surviving cells were detected when cultures were treated with concentrations of βH,7β-diHCA or βH-CA higher than 5 μM. Time-course experiments showed that toxicity was evident after 2 days of incubation (Supplemental Figure 5B).

The survival-promoting effect of βH,7α-diHCA was completely blocked by coinoculation with GGPP (Figure 4G), which indicated that this effect was mediated by LXR. Conversely, the cell death effects of βH,7β-diHCA or βH-CA were not blocked by GGPP (Figure 4G), which suggested that the survival-promoting effects of βH,7α-diHCA, but not the toxic effects of βH,7β-diHCA or βH-CA, were mediated by LXR.

We next examined whether the toxic effects of βH,7β-diHCA or βH-CA could be mediated by receptor blockade or competition. We first performed a binding and activation FRET assay using βH,7α-diHCA at its most effective concentration and increasing concentrations of βH,7β-diHCA or βH-CA. We found that βH,7β-diHCA and βH-CA reduced the binding of βH,7α-diHCA in a dose-dependent manner (Figure 5A), which indicates that these toxic cholestenoic acids compete with the neuroprotective βH,7α-diHCA for binding to the LXRβ-LBD. Kᵣ values were 0.91 μM for βH-CA and 1.22 μM for βH,7β-diHCA.

Furthermore, we performed competition experiments using the known LXR ligand 24,25-EC (at its most effective concentration) and increasing concentrations of each of the cholestenoic acids studied. βH,7α-diHCA, βH-CA, and βH,7β-diHCA each reduced the binding of 24,25-EC to the LXRβ-LBD in a dose-dependent manner (Figure 5B), which suggests that each of these cholestenoic acids competes with 24,25-EC for binding to the LXRβ-LBD. βH-CA had the lowest Kᵣ value (βH,7α-diHCA, 3.56 μM; βH,7β-diHCA, 3.45 μM; βH-CA, 2.43 μM).

In order to further prove that βH,7α-diHCA, βH-CA, and βH,7β-diHCA are specific LXR ligands in neural cells, we incubated the acids with GGPP (6). This antagonized and inhibited the ligand-dependent induction of FRET by βH,7α-diHCA, βH-CA, and βH,7β-diHCA as well as by the known LXR ligand 24,25-EC (Figure 5C), which indicates that βH,7α-diHCA, βH-CA, and βH,7β-diHCA bind to LXR. The Kᵣ values for the compounds of interest when incubated with GGPP were 3.96 μM for 24,25-EC, 1.95 μM for βH,7α-diHCA, 2.51 μM for βH,7β-diHCA, and 2.02 μM for βH-CA.

We next tested whether βH,7α-diHCA could also compete and reduce the toxic effect of βH,7β-diHCA or βH-CA. Treatment with 10 μM βH,7α-diHCA reversed the loss of islet-1 cells and the increased number of activated caspase-3 cells induced by 2 μM βH,7β-diHCA or βH-CA (Figure 5, D and E). These observations reinforce the notion of balance between the survival- and death-inducing effects of cholestenoic acids and suggest a new avenue for therapeutic intervention.

βH,7α-diHCA promotes survival of rodent motor neurons in vivo and prevents toxicity by βH-CA. In order to examine whether the observed effects of cholestenoic acids on motor neuron survival could also be detected in the mammalian brain in vivo, we performed cholestenoic acid injections into the cerebral aqueduct of E11.5 mice in utero and analyzed brain sections at the midbrain level at E13.5 (Figure 5F). While only occasional double-positive islet-1 ‘active caspase-3’ cells remained at the end of the experiment (Supplemental Figure 7), reliable cell counts could be performed for islet-1 and TH (Figure 5, G–O). βH,7α-diHCA did not affect the number of TH+ neurons, but increased the number of islet-1 oculomotor neurons, indicative of a selective survival effect of βH,7α-diHCA on oculomotor neurons in vivo. Upon injection of βH-CA, the number of islet-1 oculomotor neurons was reduced, showing the toxic effect of this metabolite on motor neurons in vivo. Finally, coinjection of βH-CA and βH,7α-diHCA reversed the loss of oculomotor neurons induced by βH-CA, indicating that βH,7α-diHCA protects oculomotor neurons against cholestenoic acids such as βH-CA in vivo. These data further confirm the specificity of the neuronal survival and toxic effects of these cholestenoic acids on islet-1 neurons, but not TH+ neurons, as well as the competitive interaction between prosurvival and toxic cholestenoic acids on motor neurons in vivo.

In summary, our results indicate that cholestenoic acids regulate the number of islet-1 motor neurons by controlling islet-1 expression (βH,7O-CA) and competitively regulating neuronal survival in a positive (βH,7α-diHCA) or negative (βH,7β-diHCA and βH-CA) manner.

Discussion

The results presented herein show that cholestenoic acids are not mere intermediate metabolites of bile acid biosynthesis, but rather represent a diverse family of bioactive compounds capable of regulating nuclear receptor function. Cholestenoic acids were found to specifically activate LXR and elicit an exquisite array of functions, ranging from the regulation of islet-1 expression to the positive and negative regulation of motor neuron survival both in vitro and in vivo. Moreover, our findings identified cholestenoic acids in human CSF to be deregulated in plasma of CTX and SPG5 patients. Importantly, an absence of neuroprotective cholestenoic acids was found in CTX patient plasma, while a combination of decreased neuroprotective and increased toxic cholestenoic acids was detected in SPG5 plasma. These results are consistent with cholestenoic acids being key regulators of motor neuron function in development and disease.

Cholesterol is present at high levels in the CNS of vertebrates and is metabolized in brain predominantly to 24S-HC (42), which accounts for about two-thirds of brain cholesterol metabolism (43). Low levels of 26-HC have been found in human and mouse brain (Supplemental Results and Supplemental Table 4) and in human CSF, where it may be imported from the blood (25, 44–46). Conversely, 7αH,30-CA, a metabolic product of 26-HC, is exported from brain to blood in humans (26). In the current study, we found 4 intermediates in the biosynthesis of 7αH,30-CA from 26-HC via CYP27A1 and CYP7B1 to be present in normal CSF: βH-CA, βH,7α-diHCA, 7α,26-diHCH, and 7α,26-diHCO (Figure 1A). While βH-CA and 7α,26-diHCO were previously found in human neu-
ral tissue (46, 47), 3β,7α-diHCA and 7α,26-diHCO have not been previously found in neural tissue or CSF. Importantly, the identification of these intermediates in the biosynthesis of 7αH,3O-CA lends further support to the hypothesis that 7αH,3O-CA is biosynthesized in the human brain (26). This pathway is also conserved in rodents, in which 26-HC is the precursor for the synthesis of 3β-HCA in fetal neurons and of 3β,7α-diHCA and 7αH,3O-CA in fetal astrocytes (Figure 1A and ref. 48). There have been few studies of specific brain regions for cholestenoic acids; however, human retina has been found to contain 3β-HCA at a level of up to 130 pmol/mg protein, about 10% of the level of 24S-HC in brain gray matter (47), whereas in chronic subdural hematoma, 7αH,3O-CA has been found at a level of 1.5 μM (49). Future studies will be directed at profiling cholestenoic acids in distinct brain regions. Surprisingly, Cyp7b1−/− and Cyp27a1−/− mice, unlike human SPG5 patients and some CTX patients, do not suffer from motor neuron disease, despite an absence of 3β,7α-diHCA in brain and plasma (Supplemental Table 4). These findings suggest that other LXR ligands may compensate for the accumulative effect of altered levels of cholestenoic acids in Cyp7b1−/− and Cyp27a1−/− mice.

Classic studies by Lehmann et al. (4) and Janowski et al. (3) defined the general structural requirements of steroidal LXR ligands to be a 3β-hydroxy-5-ene function in the ring system and a hydroxy, oxo, or epoxide function on the C-17 side-chain (3, 4). The side-chain functions have more recently been extended to include a carboxylic acid group (7, 50), a functional group that is also present in the synthetic nonsteroidal LXR ligand GW3965. In the current study, we confirmed the LXR-activational capacity of cholestenoic acids with a 3β-hydroxy-5-ene structure in neural cells and showed that despite the introduction of a 7α-hydroxy, 7β-hydroxy, or 7-oxo group, LXR activity was maintained. On the contrary, the 3-oxo-4-ene equivalents of these acids were not LXR ligands. Thus, 3β-HCA, 3β,7α-diHCA, its 7β-isomer (3β,7β-diHCA), and the necessary 7-oxo intermediate in the epimerization reaction (3βH,7O-CA) are all LXR ligands effective at micromolar concentrations. Moreover, none of these acids were found to activate FXR, VDR, or NRUR1 in neural cells, thereby confirming the specificity of their effect on LXR.

Numerous studies in recent years have linked LXR to neuronal degeneration (2, 5, 51–53). These studies have used Lxrb−/− and Lxra−/−Lxrb−/− mice. Indeed, both LXR isoforms are expressed in brain (1, 54), and the knockout mice show progressive lipid accumulation in brain, abnormal BBB, increased reactive microglia, astrogliosis, and degeneration of adult spinal cord motor neurons (2, 5). Interestingly, a decrease in the number of oculomotor neurons was also observed in mice with deletion of Lxra. In embryonic brain in utero, LXR ligands were found to cause cell death, and the latter was present at high levels in patients with SPG5. Instead, 3β,7α-diHCA, which was found at low levels in
Spg5 and absent in CTX patients, promoted motor neuron survival, while 3βH,7α-CA regulated islet-1 expression levels. Thus, our results uncovered several novel functions of cholestenolic acids and identified them as LXR ligands and key regulators of motor neuron function in development and disease. We suggest that factors with neuroprotective function, such as 3β,7α-dihC4A, may thus find a therapeutic application to prevent motor neuron loss. This suggestion is supported by our finding that 3β,7α-dihC4A, a cholestenolic acid that decreased in Spg5, prevented cell loss by 3β-HCA, a cholestenolic acid that accumulates in Spg5.

Methods

Extraction of sterols. Sterols were extracted from CSF, plasma, or mouse brain into ethanol and fractionated by reversed phase solid phase extraction (SPE) to give a cholestenolic acid– and oxysterol-rich fraction devoid of cholesterol (7, 55, 56).

Charge-tagging of sterols. The sterols were charge-tagged with the GP-hydrazine as described previously (7, 55, 56). This greatly enhances their response when analyzed by LC-ESI-MS and tandem mass spectrometry (MSn).

Reagents. HPLC-grade water and solvents were from Fisher Scientific or Sigma-Aldrich. Authentic sterols, steroids, cholestenolic acids, bile acids, and their precursors were from Avanti Polar Lipids, Steraloids, Sigma-Aldrich, or from previous studies in our laboratories. Girard P (GP) reagent [1-(carboxymethyl)pyridinium chloride hydrate] was from TCI Europe or synthesized in earlier studies, and cholesterole oxidase from Streptomyces sp. was from Sigma-Aldrich. Certified Sep-Pak C18, 200 mg SPE cartridges were from Waters. Luer-lock syringes were from BD Biosciences.

LC-ESI-MSr on the LTQ-Orbitrap. LC-ESI-MS and LC-ESI-MSn were performed using an Ultimate 3000 HPLC system (Dionex) linked to the ESI source of a LTQ-Orbitrap XL or LTQ-Orbitrap Velos (Thermo Fisher) mass spectrometer as described previously (7, 55, 56).

Luciferase reporter assay. The ability of oxysterols and their acidic metabolites to activate several nuclear receptors (i.e., LXRα, LXRβ, FXR, VDR, and NURR1) was tested in luciferase assays. Transient transfection studies were performed in the mouse neuronal cell line SN4741. This cell line was selected because the oxysterols and acidic metabolites tested were initially identified in CSF. Cells were plated in 24-well plates (5 × 105 cells/well) 24 hours before transfection and transfected with 1 μg plasmid DNA/well complexed with 2 μl Lipofectamine 2000 (Invitrogen). Cells were transfected with 400 ng of a LXRα, FXRα, VDRα, or NURR1-responsive luciferase reporter construct and 200 ng LXRα, FXRβ, VDR, or NURR1. A reporter gene expressing the Renilla luciferase (pRL-TK; Promega) and the Society for Neuroscience. Lxra−/−/Lxrβ−/− mouse cell cultures were from the colony at the Department of Biosciences and Nutrition at Novum, Karolinska Institutet. Male and female WT and Lxra−/−/Lxrβ−/− mice were generated as previously described (59, 60). Mice were backcrossed onto a C57BL/6 background for 10 generations. Male Cyp7b1−/− mouse brain and plasma were from animals generated at the University of Edinburgh. Male mice homozygous for targeted disruption of the Cyp7b1 gene (61) congenic on the C57BL/6 genetic background (>15 generations backcrossed to C57BL/6) and WT littermate controls were generated from Cyp7b1−/− crosses. Male Cyp27a1−/− mouse tissue and plasma was purchased from The Jackson Laboratory (strain B6.129-Cyp27a1−/−/J; ref. 62). The Cyp27a1−/− colony was backcrossed to C57BL/6J inbred mice for approximately
12 generations by the donating investigator (62) prior to sending to The Jackson Laboratory Repository. Upon arrival, mice were bred to C57BL/6j inbred mice for at least 1 generation to establish the colony. WT animals from the colony were used as controls. Tissue and blood sampling from these mice was performed under the aegis of the UK Scientific Procedures (Animals) Act, 1986.

**Primary midbrain cultures.** Brains from E11.5 mice were obtained, and the midbrain region was dissected, mechanically dissociated, plated on poly-D-lysine (150,000 cells/cm²), and grown in serum-free N2 media consisting of F12/DMEM (1:1 mixture) with 10 ng/ml insulin, 100 μg/ml apo-transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 6 mg/ml glucose, and 1 mg/ml BSA. Cells were treated for 3 days in vitro with the compounds of interest, fixed with 4% PFA, and processed for staining using appropriate antibodies. Human samples, collected according to the principles of the Declaration of Helsinki, were provided to University Hospital Basel, Barts Health NHS Trust, St. Mary’s Hospital, Institute of Child Health, Connegoiano Research Centre, Federico II University, University of Tubingen, Kurume University School of Medicine, and University Hospital “Attikon” with written informed consent and IRB and ethical approval. Ethical approval was granted by Stockholm Norra Djurförsöksets Nämnd for zebrafish experimentation (nos. N293/09 and N338/10), mouse experimentation (nos. N154/06, N145/09, N370/09, and N273/11), and in utero experimentation (no. N486/12).

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Address correspondence to: William J. Griffiths, College of Medicine, Grove Building, Swansea University, Singleton Park, Swansea SA2 8PP, United Kingdom. Phone: 44.1792295562; E-mail: wj.griffiths@swansea.ac.uk. Or to: Yuqin Wang, College of Medi-


