Identification of a novel sulfonated oxysterol, 5-cholesten-3β,25-diol 3-sulfonate, in hepatocyte nuclei and mitochondria

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Abstract **This study reports the discovery of a novel sulfonated oxysterol found at high levels in the mitochondria and nuclei of primary rat hepatocytes after overexpression of the gene encoding steroidogenic acute regulatory protein (StarD1). Forty-eight hours after infection of primary rat hepatocytes with recombinant adenovirus encoding StarD1, rates of bile acid synthesis increased by 4-fold. Concurrently, [^{14}C]cholesterol metabolites (oxysterols) were increased dramatically in both the mitochondria and nuclei of StarD1-overexpressing cells, but not in culture medium. A water-soluble [^{14}C]oxysterol product was isolated and purified by chemical extraction and reverse-phase HPLC. Enzymatic digestion, HPLC, and tandem mass spectrometry analysis identified the water-soluble oxysterol as 5-cholesten-3β,25-diol 3-sulfonate. Further experiments detected this cholesterol metabolite in the nuclei of normal human liver tissues. Based upon these observations, we hypothesized a new pathway by which cholesterol is metabolized in the mitochondrion.**—Ren, S., P. Hylemon, Z-P. Zhang, D. Rodriguez-Agudo, D. Marques, X. Li, H. Zhou, G. Gil, and W. M. Pandak. **Identification of a novel sulfonated oxysterol, 5-cholesten-3β,25-diol 3-sulfonate, in hepatocyte nuclei and mitochondria. *J. Lipid Res.* 2006.** 47: **1081–1090.**

Supplementary key words nucleus • steroidogenic acute regulatory protein • cholesterol transporter • bile acids • cholesterol metabolism • nuclear oxysterol ligands

The metabolism of cholesterol to primary bile acids occurs via two main pathways in hepatocytes (1). The "neutral" pathway is considered the major pathway at least in humans and rats (2). The highly regulated microsomal cholesterol 7α-hydroxylase (CYP7A1) is the first and rate-limiting step of this pathway (3). An alternative pathway, the "acidic" pathway, is initiated by mitochondrial cholesterol 27-hydroxylase (CYP27A1). Oxysterol intermediates of the acidic pathway, such as 25- and 27-hydroxycholesterol, have been shown to be regulators of cholesterol homeostasis (4). These oxysterols represent regulatory molecules for the expression of many other genes encoding enzymes involved in cholesterol biosynthesis and transport (5–7). Increased CYP27A1 activity in peripheral tissues may both downregulate cholesterol synthesis through the sterol-regulatory element binding protein pathway and enhance the efflux of cholesterol and its elimination via liver sterol receptor (LXR) (8). However, the physiological and authentic in vivo LXR ligand is currently unknown (9). Thus, characterizing endogenous oxysterols and their mechanism of action is critical for a better understanding of sterol homeostasis.

Overexpression of CYP27A1 in hepatocyte culture results in minimal increases (~50%) in the rates of bile acid biosynthesis, compared with the 7-fold increases seen after overexpression of CYP7A1 (10). This led us to hypothesize that cholesterol delivery to the mitochondria, where CYP27A1 is located, may be rate-determining for bile acid synthesis via the acidic pathway. A mitochondrial cholesterol delivery protein, steroidogenic acute regulatory protein (StarD1), has been detected in liver tissues and hepatocytes. Its expression is highly regulated, like the StarD1 found in steroidogenetic tissues (11). Recently, we found that overexpression of StarD1 dramatically increases cholesterol catabolism to bile acids both in cultured primary hepatocytes and in vivo (12, 13). Overexpression of StarD1 in vivo not only increases the rates of bile acid synthesis to the same level as overexpression of CYP7A1 but also produces a similar composition of bile acids in vivo (13). Thus, it is reasonable to hypothesize that oxysterols

Abbreviations: CYP27A1, cholesterol 27-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; HST2, hydroxycholesterol sulfonate transferase 2; LXR, liver sterol receptor; StarD1, steroidogenic acute regulatory protein.

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generated in the mitochondria may play an important role in the maintenance of intracellular cholesterol homeostasis. In this study, we present evidence for the increased formation and identification of a sulfonated oxysterol in primary rat hepatocytes after overexpression of the gene encoding StarD1.43

MATERIALS AND METHODS

Cell culture reagents and supplies were purchased from GIBCO BRL (Grand Island, NY). [^{14}C]cholesterol and [^{3}H]25-hydroxycholesterol were from New England Nuclear (Boston, MA). [^{14}C]27-hydroxycholesterol was prepared as described previously (14). Cyclodextrin was purchased from Cyclodextrin Technologies Development, Inc. (Gainesville, FL). Normal human liver tissues were from a National Institutes of Health-approved facility, the Liver Tissue Procurement Distribution System, University of Minnesota. All other reagents were from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise.

Adenovirus preparation and propagation

The adenovirus construct used in this study was obtained through the Massey Cancer Center Shared Resource Facility of the Virginia Commonwealth University, as described previously (15).

Culture and subcellular fractionation of primary rat hepatocytes and lipid fractionation

Primary rat hepatocyte cultures, prepared as described previously (16), were plated on 150 mm tissue culture dishes ($\sim$2.5 $\times$ 10^7 cells) in Williams' E medium containing dexamethasone (0.1 μM). Cells were maintained in the absence of thyroid hormone. Twenty-four hours after plating, culture medium was removed, and 4 ml of fresh medium was added. Cells were then infected with recombinant adenovirus encoding either the StarD1 (Ad-CMV-StarD1) or the CYP7A1 (Ad-CMV-CYP7A1) cDNA in front of the human cytomegalovirus (CMV) promoter or no cDNA, as a control virus. The viruses were allowed to incubate for 2 h in minimal culture medium with gentle shaking of the plates every 15 min. After 2 h of incubation, unbound virus was removed and replaced with 20 ml of fresh medium, and 2.5 μCi of [^{14}C]cholesterol was added. After 48 h, cells were then harvested and processed for nuclear isolation as described (17), with minor modifications (**Fig. 1**). Briefly, cells were disrupted by Dounce homogenization in buffer A (10 mM HEPES-KOH, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM sodium EDTA, and 1 mM EGTA) and spun at 1,000 *g* for 10 min. The nuclear pellet was further fractionated by resuspension in 2.5 ml of a 1:1 mixture of buffer A and buffer B (2.4 M sucrose, 15 mM KCl, 2 mM sodium EDTA, 0.15 mM spermine, 0.15 mM spermidine, and 0.5 mM dithiothreitol) and centrifuged at 100,000 *g* for 1 h at 4°C through a 1 ml cushion of a 3:7 mixture of buffers A and B. The washed nuclear pellet was resuspended in buffer A containing 0.5% (v/v) Nonidet P-40 and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant is designed as Nonidet P-40 extracts (fraction C), and the pellets are designed as purified nuclei (fraction D).

The purified nuclei (fraction D) were resuspended and digested by 2 mg/ml DNase I in 50 mM acetic buffer, pH 5.0, and 10 mM $MgCl_2$ at 37°C for 2 h. After centrifugation at 10,000 *g* for 20 min, the supernatant was designed as DNase digests (fraction E). The pellets were further digested by 2 mg/ml proteinase K in PBS at 50°C for 16 h, and the solution was designed as nuclear protease digests (fraction F). Total lipids in each fraction were extracted by adding 3.3 volumes of chloroform-methanol (1:1) and separated into two phases, methanol/water and chloroform phases, as described previously (18). The counts of [^{14}C]cholesterol/cholesterol derivatives in the methanol/water and chloroform phases were measured by liquid scintillation counting.

Mitochondria were isolated from primary hepatocytes and human liver tissues as described previously (19). NADP-linked isocitrate dehydrogenase activity was used as a mitochondrial marker.

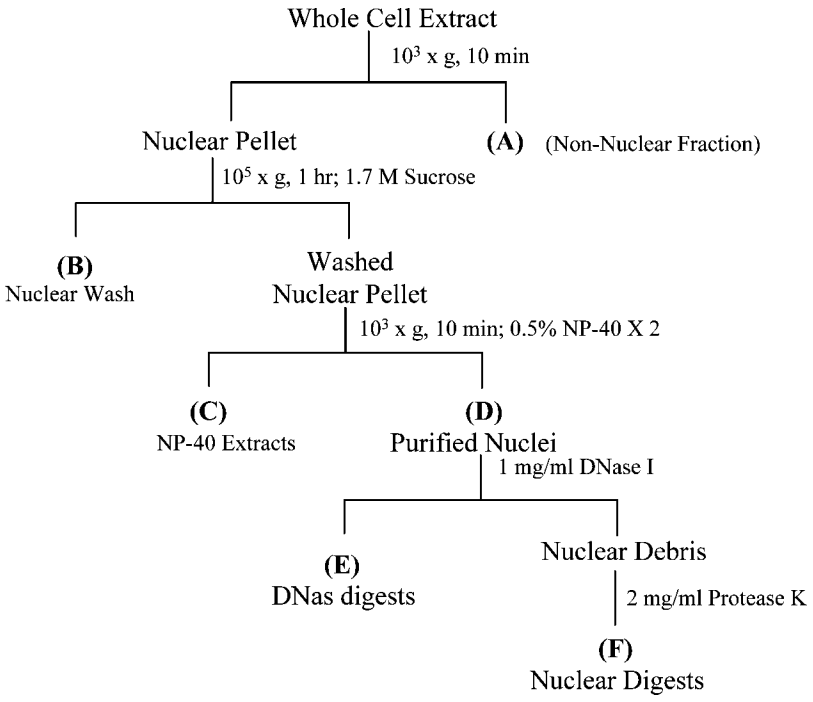

Fig. 1. Subcellular fractionation protocol. For details, see Materials and Methods. NP-40, Nonidet P-40.

Glucose 6-phosphatase activity, a microsomal marker, was used to monitor the purity of the isolated mitochondrial fractions.

HPLC analysis of cholesterol derivatives

[^{14}C]cholesterol derivatives in the chloroform phase were analyzed by HPLC on an Ultrasphere Silica column (5 μm × 4.6 mm × 25 cm; Beckman) using the HP Series 1100 solvent delivery system (Hewlett-Packard) at a flow rate of 1.3 ml/min. The column was equilibrated and run in a solvent system of hexane-isopropanol-glacial acetic acid (965:25:10, v/v/v) as the mobile phase. The effluents were collected every 0.5 min (0.65 ml per fraction), except as indicated. The counts of [^{14}C]cholesterol/cholesterol derivatives were determined by scintillation counting. The column was calibrated with [^{14}C]cholesterol, [^{3}H]25-hydroxycholesterol, and [^{14}C]27-hydroxycholesterol.

Total [^{14}C]cholesterol derivatives found in methanol/water phases were analyzed on an Ultrasphere PTH C-18 column (5 μm × 4.6 mm × 25 cm; Beckman) at a flow rate of 0.8 ml/min. The column was equilibrated and run in 20 mM KH_2PO_4, pH 4.2-acetonitrile-methanol (1:3:6, v/v/v) as the mobile phase. The effluents were monitored at 195 nm and collected every 0.5 min (0.4 ml per fraction), except as indicated. The column was calibrated with tauroursodeoxycholic acid, glycoursodeoxycholic acid, taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, and progesterone as standards.

Sulfatase treatment of the purified nuclear [^{14}C]cholesterol derivatives

The purified nuclear [^{14}C]cholesterol derivatives were digested with 2 mg/ml sulfatase (EC 3.1.6.1) (Sigma) in 50 mM acetic buffer, pH 5.0, by incubation at 37°C for 4 h. The products were extracted into chloroform phase from the methanol/water phase by adding 3.3 volumes of methanol-chloroform (1:1, v/v) to the reaction mixture. [^{14}C]cholesterol derivatives in both the chloroform and methanol/water phases were then analyzed by HPLC as described above.

Reverse-phase liquid chromatography/tandem mass spectrometry analysis of nuclear cholesterol derivatives

Purified samples to be analyzed were constituted in methanol-water (20:80, v/v) and separated on a ThermoKeystone Aquasil C18 column (5 μm, 2.1 mm × 100 mm). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The 20 min gradient was as follows: 0–10.0 min, 10–95% B linear; 10.0–15.0 min, 95% B; 15.0–15.1 min, 95–10% B linear; 15.1–20.0 min, 10% B. The elution stream (0.3 ml/min) from the HPLC apparatus was introduced into an ABI 4000 Triple Quadruple Mass Spectrometer (MDS Sciex, Toronto, Canada) with a Turbo Ion Spray ionization source for the analyses. The mass spectrometer was operated in negative ion mode, and data were acquired using both full-scan mode and the product ion mode for tandem mass spectrometry (MS/MS). The optimized parameters for Q1 full scan under the negative mode were as follows: CUR, 10; GS1, 40; GS2, 40; TEM, 400; IS, −4,500; DP, −150; EP, −10. The optimized parameters for the product scan of *m/z* 481 under the negative mode were as follows: CUR, 10; GS1, 40; GS2, 40; TEM, 400; IS, −4,500; CAD, 5; DP, −150; EP, −10; CE, 50; CXP, −15.

TABLE 1. Distribution of [^{14}C]cholesterol derivatives in primary rat hepatocytes after StarD1or CYP7A1 overexpression

	Subcellular Fraction				
	Nonnuclear		Nuclear		
Adenovirus	A	B	C	E	F
Control	43 ± 2	2.7 ± 5	47 ± 3	1.0 ± 0.2	7 ± 3
StarD1	38 ± 8	3.1 ± 2	46 ± 6	1.7 ± 0.1	10 ± 2
CYP7A1	43 ± 3	2.9 ± 4	49 ± 8	1.1 ± 0.2	8 ± 2

CYP7A1, cholesterol 7α-hydroxylase; StarD1, steroidogenic acute regulatory protein. [^{14}C]cholesterol was added at 2 h after adenovirus infection, and cells were harvested 48 h later. Subcellular fractions were prepared as described in Materials and Methods. An aliquot from each fraction was taken for liquid scintillation counting. Values represent means of three experiments ± SD of the percentage of radioactivity found in each fraction with respect to the total radioactivity found in all fractions.

Statistics

Data are reported as means ± SD. Where indicated, data were subjected to *t*-test analysis and determined to be significantly different at $P < 0.05$.

RESULTS

A novel nuclear oxysterol is synthesized in mitochondria of primary rat hepatocytes upon StarD1 overexpression

Primary rat hepatocytes were infected with recombinant adenovirus encoding either StarD1 or CYP7A1, as de-

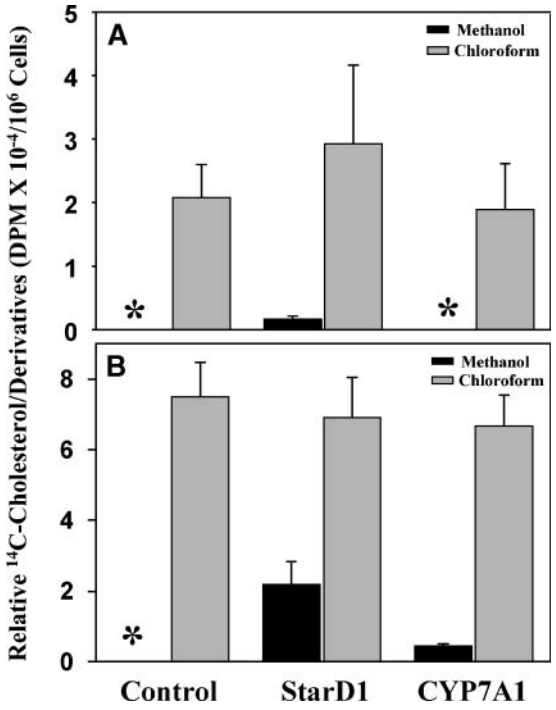

Fig. 2. Phase distribution of [^{14}C]cholesterol derivatives in nuclei of primary rat hepatocytes after overexpression of steroidogenic acute regulatory protein (StarD1) and cholesterol 7α-hydroxylase (CYP7A1). Rat primary hepatocytes were infected with the indicated viruses. Forty-eight hours later, cells were harvested and subcellular fractions were prepared. Fractions E and F were processed for lipid analysis as explained in Materials and Methods. Top: DNase digests (fraction E). Bottom: Nuclear digests (fraction F). * Counts of [^{14}C]cholesterol derivatives were not detected. The values represent means ± SD (n = 5).

scribed in Materials and Methods. Forty-eight hours after infection, bile acid synthesis was increased by 4-fold and 7-fold, respectively, as reported previously (18). Cells infected with control virus had similar levels of bile acid synthesis as uninfected cells.

The subcellular distribution of [^{14}C]cholesterol metabolites formed 48 h after virus infection is summarized in **Table 1**. The isolated mitochondrial fractions did not show any detectable glucose 6-phosphatase activity, suggesting that the mitochondrial fractions were not contaminated by endoplasmic reticulum. The nuclear fractions were monitored by Giemsa staining under light microscopy. The purity of the nuclei was >95%. Approximately 50% of the total cellular counts of [^{14}C]cholesterol metabolites were found in the nuclear fraction. The other 50% were located in nonnuclear organelles, including cytosol, plasma membranes, lysosomes, and mitochondria (fractions A and B). Only a small number of counts were detected in the DNase-digested fractions (fraction E). The total extractable [^{14}C]cholesterol metabolite count in nuclear debris from StarD1-infected cells was slightly greater than those of CYP7A1-infected or control nuclear extracts (fraction F).

Surprisingly, [^{14}C]cholesterol metabolites in the methanol/water phase extracted from the nuclear debris were increased dramatically in StarD1-overexpressing cells compared with control cells ($P < 0.001$, n = 5) or CYP7A1-overexpressing cells ($P < 0.001$, n = 5) (**Fig. 2**). It was observed that the major [^{14}C]cholesterol metabolites were found in the nuclear digests (fraction F) but not in Nonidet P-40 digests (fraction C), and little was found in DNase digests (fraction E). These [^{14}C]cholesterol metabolites could be extracted only after DNase and proteinase K treatments. These results suggested that the [^{14}C]cholesterol metabolites present inside the nucleus may be protein-bound oxysterol(s). We then proceeded to characterize the composition of the cholesterol metabolites in the different subcellular fractions of StarD1-overexpressing hepatocytes. We first performed reverse-phase HPLC analysis of [^{14}C]cholesterol metabolites in the methanol/water phase from the nuclear and nonnuclear fractions. **Figure 3** shows the elution profiles of lipid extracts in

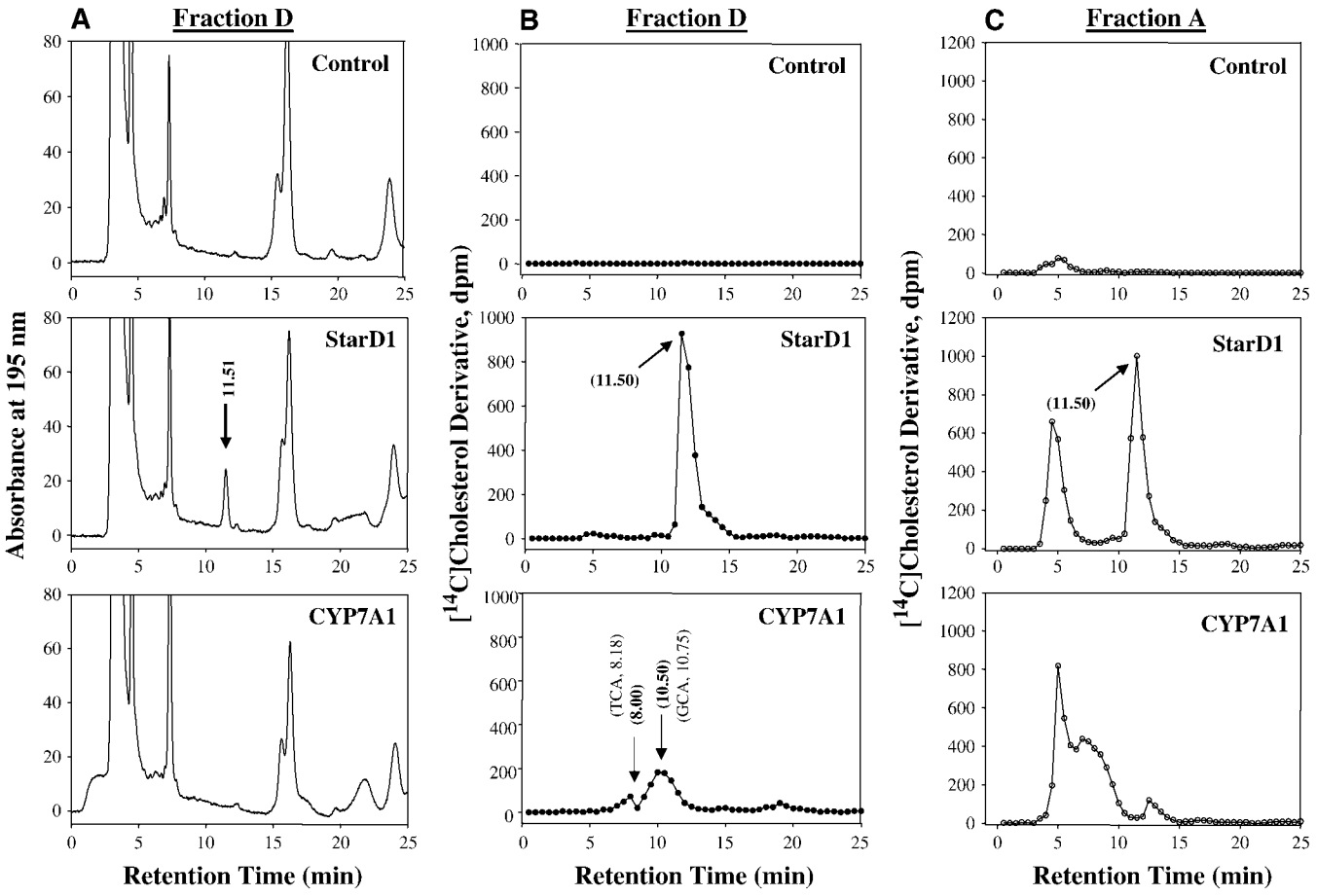

Fig. 3. HPLC analysis of [^{14}C]cholesterol derivatives in the nuclear fraction (fraction D) and nonnuclear fraction (fraction A). Twenty-four hours after the indicated recombinant adenovirus infection, cells were harvested, nuclear and nonnuclear fractions (fractions A and D) were isolated and extracted by the Folch method, and the methanol/water phase was analyzed. A: Nuclear extracts (fraction D), 195 nm profiles. B: Nuclear extracts (fraction D), ^{14}C-sterol profiles. C: Nonnuclear extracts (fraction A), ^{14}C-sterol profiles. In each case, nuclear methanol/water extracts of the equivalent of 5×10^6 cells were loaded. Each elution profile represents one of five sets of experiments (the profiles are highly repeatable).

water/methanol phases from various subcellular fractions of cells overexpressing CYP7A1 and StarD1. The elution profiles were relatively similar in the nuclear fraction (fraction D) from cells infected with the StarD1, CYP7A1, or null recombinant virus, except for an extra peak with a retention time of 11.50 min (Fig. 3A). This 11.50 min peak was the only fraction that contained ^{14}C-labeled cholesterol metabolites and was detectable only in StarD1-overexpressing cells (Fig. 3B). The nonnuclear fraction (fraction A) from the StarD1-overexpressing cells contained two major [^{14}C]cholesterol derivative peaks with retention times of 5.0 and 11.50 min. The 11.50 min peak was not detected in cells infected with either the control or the CYP7A1 virus (Fig. 3C).

Further analysis of the cholesterol metabolites in the methanol/water phases extracted from mitochondria, nuclear fractions, and culture medium after overexpression of StarD1 protein showed that two [^{14}C]cholesterol metabolites with retention times at 5.0 and 11.50 min were found in the mitochondrial fraction (**Fig. 4A, D**). Furthermore, the metabolite eluting at the 11.50 min peak was present only in mitochondria and nuclei but not in the culture medium (Fig. 4B, E), suggesting that the [^{14}C]cholesterol metabolite was synthesized in mitochondria and translocated to the nuclei. The peak with a retention time of 5.0 min was found only in mitochondria and culture medium but not in the nuclear fractions (Fig. 4C, F), suggesting that this molecule was secreted from the cells.

To characterize the chemical structure, the nuclear oxysterol was purified by reverse-phase HPLC and initially analyzed by enzymatic digestion. To determine whether the water-soluble nuclear oxysterol molecule was sulfonated, the purified metabolite was treated with sulfatase (see Materials and Methods). Products were then extracted with methanol-chloroform and characterized by HPLC analysis. After digestion of the nuclear oxysterol with sulfatase, the products of [^{14}C]cholesterol metabolites were

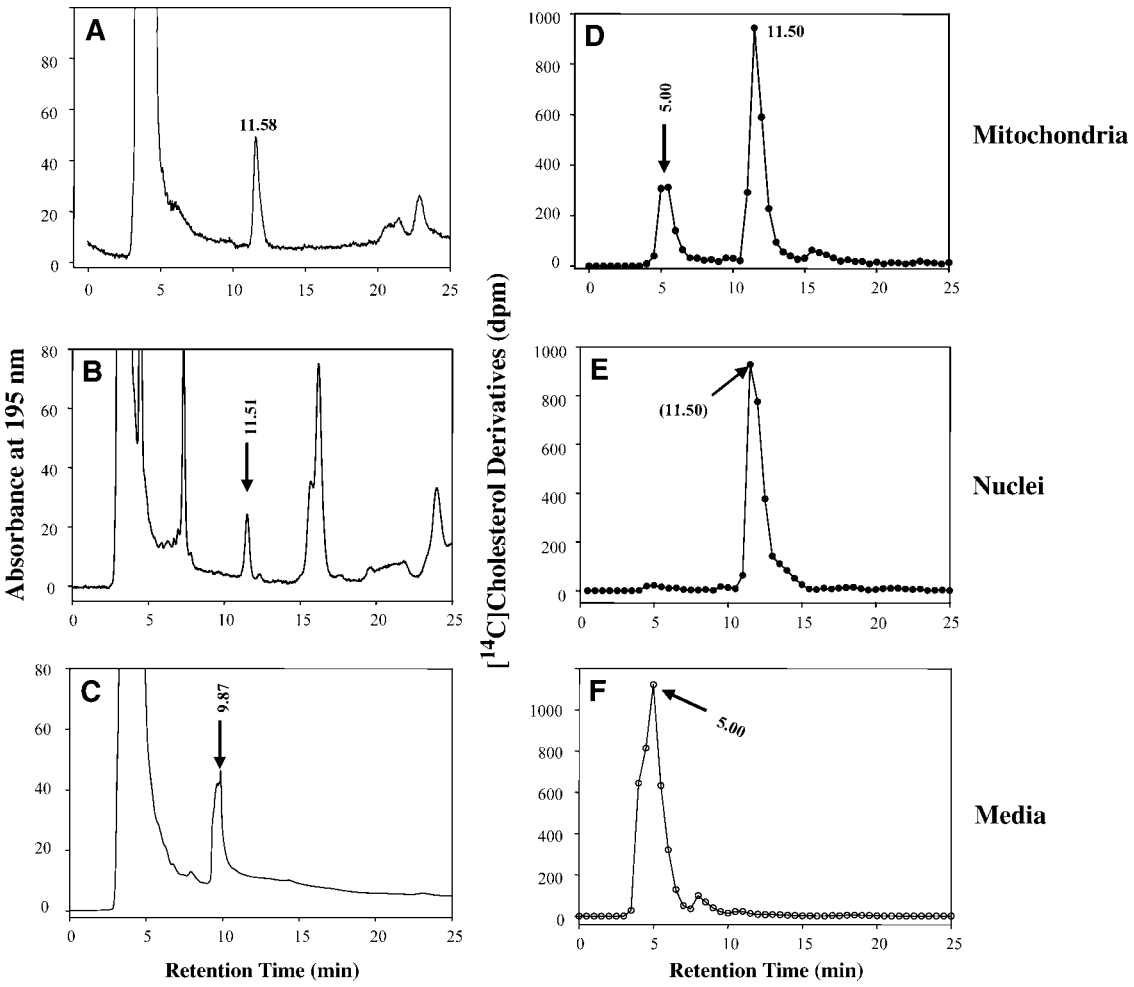

Fig. 4. HPLC analysis of the cholesterol derivatives extracted from nuclei, mitochondria, and culture medium. Rat primary hepatocytes were infected with the StarD1 adenovirus, and 2 h later, [^{14}C]cholesterol was added to the medium. Twenty-four hours after infections, cells and culture medium were harvested. Nuclei and mitochondria were isolated as described in Materials and Methods. Total lipids were extracted from the nuclei, mitochondria, and culture medium by Folch partitioning into the methanol phase and analyzed by HPLC, as described in Materials and Methods. Each elution profile represents one of five sets of experiments. A–C: 195 nm profiles. D–F: Radioactivity profiles.

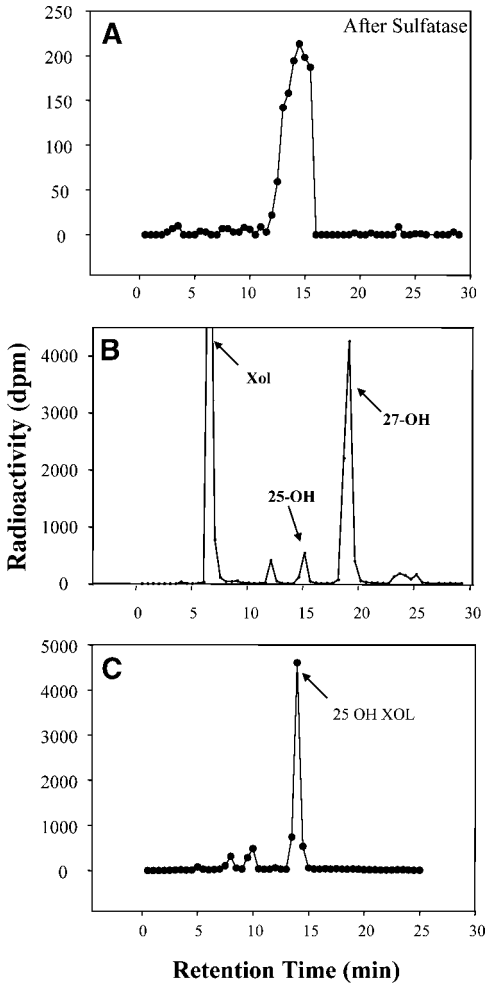

Fig. 5. Characterization of the nuclear oxysterol by enzymatic digestion followed by HPLC analysis. Nuclear [^{14}C]oxysterol derivatives were isolated from StarD1-overexpressing rat primary hepatocytes and digested with 1 mg/ml sulfatase in acetic acid buffer, pH 5.0, overnight. Total lipids were extracted with chloroform-methanol and separated by Folch partitioning. The products in the chloroform and methanol/water phases were analyzed by HPLC using a mixture of 965 ml of hexane, 25 ml of isopropanol, and 10 ml of acetic acid as mobile phase at a flow rate of 1.3 ml/min, and ^{14}C was quantified. A: HPLC elution profile of the sulfatase digestion products. B: HPLC elution profile of [^{14}C]27-hydroxycholesterol. C: HPLC elution profile of standard [^{14}C]25-hydroxycholesterol (25-OH XOL). n = 2.

extracted into the chloroform phase. HPLC analysis showed that the product has the same retention time as 25-hydroxycholesterol in our HPLC system (**Fig. 5A–C**), suggesting that the nuclear oxysterol derivative is sulfonated 25-hydroxycholesterol. TLC analysis also showed that the sulfatase-treated cholesterol metabolite migrated with the same relative mobility value as 25-hydroxycholesterol (data not shown).

To confirm the chemical structure of the nuclear oxysterol derivative, the purified nuclear sulfonated oxysterol was further analyzed by LC/MS/MS. The LC/MS/MS elution profile sorted with *m/z* 80 from a product scan spectrum of *m/z* 481 under negative ionization mode, and a prominent peak was observed at a retention time of 10.75 min, as shown in **Fig. 6A**. The LC/MS Q1 full-scan spectrum at 10.75 min showed two major molecular ions, *m/z* 480.1 and 481.5 (Fig. 6B). These two molecular ions were further analyzed by LC/MS/MS in the negative ionization mode. The product scan of *m/z* 481 at 10.75 min is shown in Fig. 6C. The characteristic fragment ions of these two molecules are very similar. Fragment ions were observed at *m/z* 80 (a), 97 (b), 107, 123 (c), 288, 465, and 59 (d) in the product scan spectrum of *m/z* 481 (Fig. 6C). These observed fragment ions indicate that the nuclear oxysterol is a sulfonated oxysterol with a sulfonate group at the 3-OH position (20) and a hydroxy group on the side chain, *m/z* 59 [molecular mass 482 = 80 (sulfonate) + 16 (O) + 386 (cholesterol)]. Combined with the data from enzymatic digestion and HPLC analysis, the nuclear oxysterol derivative was identified as 5-cholesten-3β,25-diol 3-sulfonate (25-hydroxycholesterol 3-sulfonate) (Fig. 6C). The spectra are consistent with those of previously characterized sulfated cholesterol metabolites (20). This structure is supported by high-resolution MS analysis showing its mass as 481.3118, with molecular formula $C_{27}H_{45}O_5S$ (calculated mass is 481.2988, 5.5 ppm).

Detection of this novel oxysterol in nuclei of normal human liver tissues

To determine whether this cholesterol metabolite can be detected in normal human liver, subcellular fractions were isolated from four normal human livers. The oxysterols in the methanol/water phase of each fraction were analyzed by HPLC. Interestingly, all four liver tissues showed a peak with the same retention time as 5-cholesten-3β,25-diol 3-sulfonate detected only in the nuclear fraction and not in other subcellular fractions of normal human liver tissues (**Fig. 7A**). The level of this compound is very low (~0.2 μg/g tissue) compared with those in cells (~20 μg/g cells) after StarD1 overexpression.

To confirm the structure of the nuclear oxysterol in the human liver nuclei, the peak at 11.50 min (Fig. 7A) was purified and analyzed by triple quadrupole MS/MS (Fig. 7B, C). The results show that the composition of the peak is much more complex than those purified from the culture cells (full scanning spectrum; Fig. 7B). Two of the major molecular ions, *m/z* 481 and 499 (481 + H_2O), are similar to 5-cholesten-3β,25-diol 3-sulfonate, as described above. Further analysis of the mass ions *m/z* 481 and 499 shows that the molecule has a sterol core structure with a molecular mass of ~300 kDa and one side chain with molecular ions (*m/z* 80, 97, and 107) (Fig. 7C) that are the same as the nuclear oxysterol. These results confirmed the presence of the sulfonated oxysterol in the nuclei of normal human liver tissues. The presence of the oxysterol in normal human liver nuclei suggests that the oxysterol may have physiological significance.

DISCUSSION

In this study, we report for the first time the existence of the novel nuclear oxysterol, 5-cholesten-3β,25-diol 3-

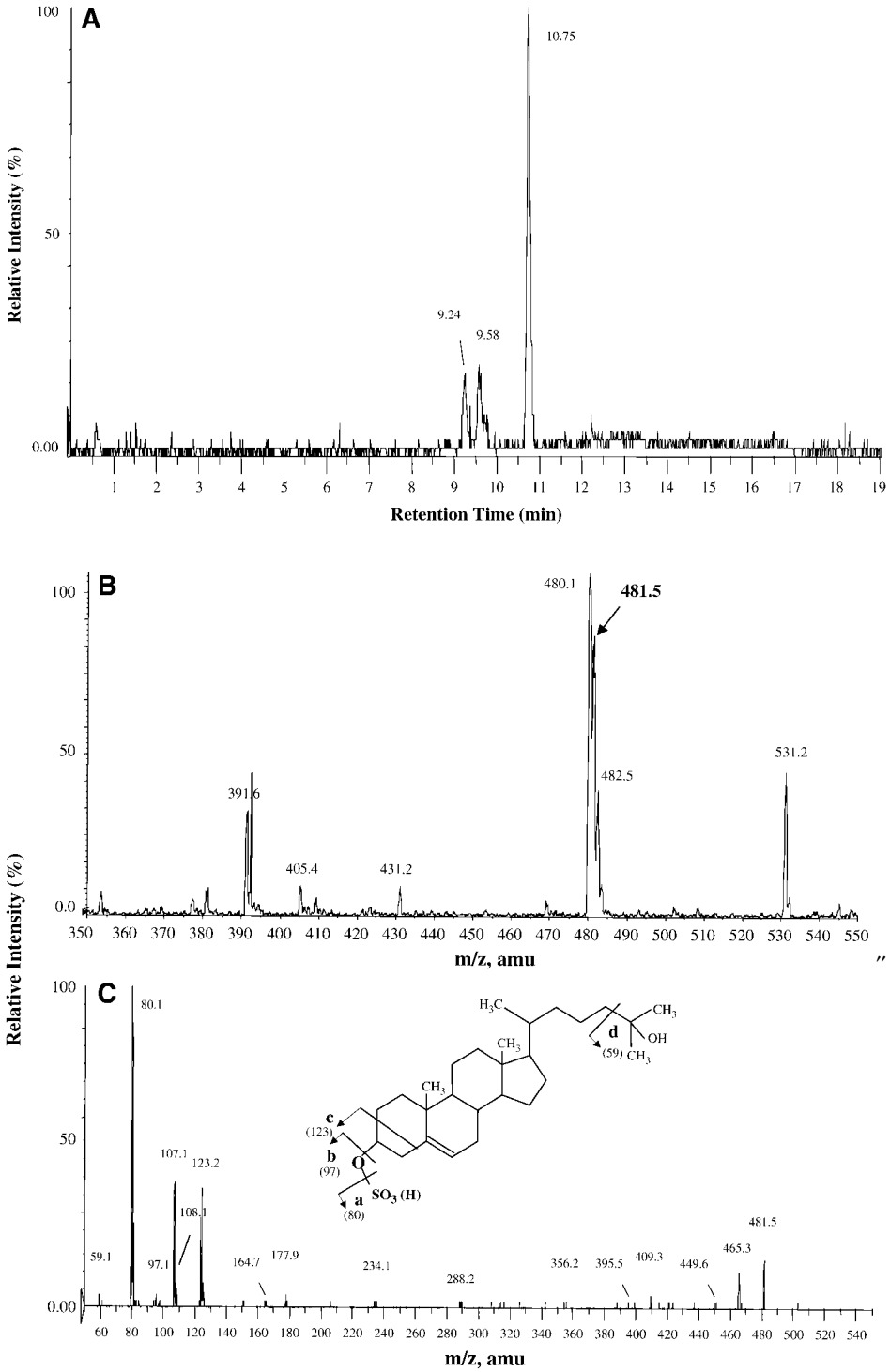

Fig. 6. Characterization of nuclear oxysterol as 5-cholesten-3β,25-diol 3-sulfonate by negative ion-triple quadruple mass spectrometry. A: HPLC/tandem mass spectrometry (MS/MS) elution profile sorted with mass ion *m/z* 80 from product scan spectrum of *m/z* 481. B: HPLC/MS negative full-scan spectrum at retention time of 10.75 min. C: HPLC/MS/MS product scan spectrum of *m/z* 481 at retention time of 10.75 min.

sulfonate, in nuclei of primary rat hepatocytes and normal human liver tissue. The levels of this oxysterol could be increased dramatically in the mitochondria and nuclei after overexpression of the mitochondrial cholesterol delivery protein, StarD1, in primary rat hepatocytes. These results give rise to a new hypothesis that StarD1 could serve as a sensor of intracellular cholesterol levels. When cholesterol levels increase, StarD1 protein delivers cholesterol into mitochondria, where it may be metabolized to 25-hydroxycholesterol, 27-hydroxycholesterol, and

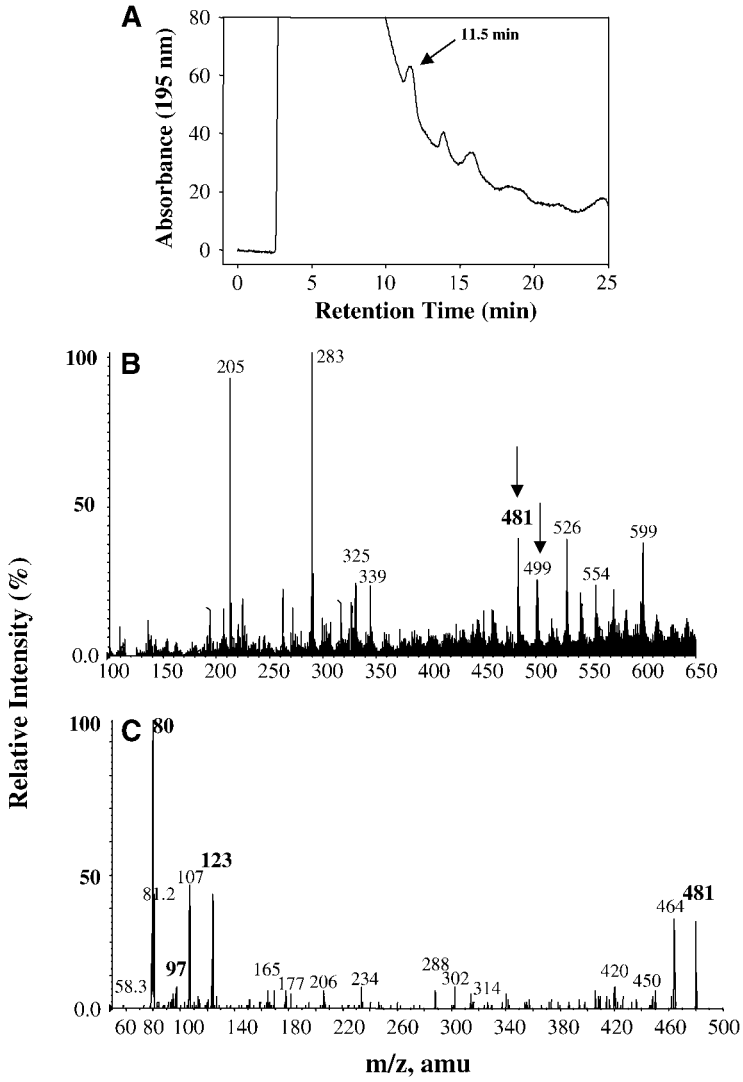

Fig. 7. HPLC analysis of oxysterols in methanol/water phases extracted from normal human liver tissues. Total lipids were extracted from the nuclear fractions, and oxysterols were extracted by Folch partitioning into methanol/water phase and analyzed by HPLC using an Ultrasphere C-18 column. A: The effluents were monitored at 195 nm by negative ion-triple quadrupole LC/MS/MS. B: Full negative scanning spectrum of the purified oxysterol peak with retention time 11.5 min by HPLC, where too small mass ions (m/z 481 and 499) fit nuclear oxysterol. C: MS/MS product scanning spectrum of m/z 481 and its fragmentation diagrams (the product spectrum of mass ion m/z 499 is similar to that of m/z 481; data not shown). Each fragment represents a negative ion of the chemical structure fragments. Based on the mass information, the structure of this nuclear oxysterol from normal human liver nuclei is the same as that described above as 5-cholesten-3β,25-diol 3-sulfonate.

5-cholesten-3β,25-diol 3-sulfonate. These cholesterol metabolites are also found in the nuclei and may be involved in the regulation of gene expression.

25-Hydroxycholesterol has been reported to be a ligand of LXRs and is believed to play an important role in the regulation of cholesterol degradation and secretion (5, 7, 21–23). Known genes regulated by LXRs include CYP7A1, ABCA1, ABCG5, and ABCG8. 25-Hydroxycholesterol has also been reported to inhibit HMG receptor and cholesterol biosynthesis by inhibiting the activation of sterol-regulatory element binding proteins (5). However, 25-hydroxycholesterol is not water-soluble, and its metabolism and physiological significance are not clear (9, 24). In contrast, the sulfonated derivative of 25-hydroxycholesterol is water-soluble. It has been reported that sulfonated steroid hormones exhibit strong steroid hormone-like activity (25). Therefore, it is possible that the nuclear sulfonated oxysterol could be a potent regulator of genes involved in cholesterol homeostasis. However, the possible nuclear receptor for this metabolite has yet to be investigated.

Several sulfonated sterols have been reported to be widely distributed in steroidogenic tissues (26) and to circulate in plasma at concentrations ranging from 328 to 924 μg/100 ml, with a blood production rate of 35–163 mg/day (27). A similar sulfonated oxysterol derivative, 24-hydroxycholeterol 3-sulfonate 24-glucuronide (based on MS/MS analysis), was reported in the serum and urine of children with severe cholestatic liver diseases (26). The 3-sulfonate of 24-hydroxycholesterol is the major hydroxycholesterol sulfonate found in meconium and infant feces (28, 29) and most likely excreted in the bile. Sulfonated sterols have been implicated in a wide variety of biological processes, such as the regulation of cholesterol synthesis, sperm capacitation, thrombin and plasmin activities, and activation of protein kinase C isoenzymes (30). Furthermore, sulfonated sterols can serve as a substrate for adrenal and ovarian steroidogenesis (31, 32). Sulfonated sterols also play an important, but unclear, role in the normal development and physiology of the skin, for which an epidermal sterol sulfonate cycle has been described (30).

This report shows the presence of 25-hydroxycholesterol 3-sulfonate in both the mitochondria and the nucleus, but not in culture medium, indicating that mitochondrial sterol hydroxylases and hydroxycholesterol sulfonate trans-

ferase 2 (HST2) may be involved in the synthesis of this oxysterol metabolite. To synthesize this molecule, two reactions must be involved in the generation of the nuclear oxysterol: 25-hydroxylation and 3β-sulfonation. It is not clear at present whether sterol 27-hydroxylase is responsible for the biosynthesis of 25-hydroxycholesterol, but it is a possibility. We hypothesize that the first step should be 25-hydroxylation of cholesterol before sulfonation at the 3β position. To date, hydroxycholesterol sulfotransferases have not been investigated in liver tissues, although two isoenzymes (HST2a and HST2b) have been cloned and identified in steroidogenic and skin tissues (30, 33). 25-Hydroxycholesterol 3-sulfonate may be further metabolized via glucuronidation and secretion via the bile in a manner similar to 3β-sulfonate cholesterol 24-glucuronide (26). However, it is not clear at present that the [^{14}C]cholesterol derivative with retention time of 5.0 min is the product of glucuronidation of 25-hydroxycholesterol 3-sulfonate (Figs. 3, 4). Further studies are in progress to elucidate the mechanism(s) underlying the role of this novel nuclear sulfonated oxysterol in the maintenance of intracellular cholesterol homeostasis.

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