Oxysterols are substrates for cholesterol sulfotransferase

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Abstract Oxysterols constitute a class of cholesterol derivatives that exhibit broad biological effects ranging from cytotoxicity to regulation of nuclear receptors. The role of oxysterols such as 7-ketocholesterol (7-KC) in the development of retinal macular degeneration and atheromatous lesions is of particular interest, but little is known of their metabolic fate. We establish that the steroid/sterol sulfotransferase SULT2B1b, known to efficiently sulfonate cholesterol, also effectively sulfonates a variety of oxysterols, including 7-KC. The cytotoxic effect of 7-KC on 293T cells was attenuated when these cells, which do not express SULT2B1b, were transfected with SULT2B1b cDNA. Importantly, protection from 7-KC-induced loss of cell viability with transfection correlated with the synthesis of SULT2B1b protein and the production of the 7-KC sulfoconjugate (7-KCS). Moreover, when 7-KCS was added to the culture medium of 293T cells in amounts equimolar to 7-KC, no loss of cell viability occurred. Additionally, MCF-7 cells, which highly express SULT2B1b, were significantly more resistant to the cytotoxic effect of 7-KC. We extended the range of oxysterol substrates for SULT2B1b to include 7α/7β-hydroxycholesterol and 5α,6α/5β,6β-epoxycholesterol as well as the 7α-hydroperoxide derivative of cholesterol. Thus, SULT2B1b, by acting on a variety of oxysterols, offers a potential pathway for modulating in vivo the injurious effects of these compounds.—Fuda, H., N. B. Javitt, K. Mitamura, S. Ikegawa, and C. A. Strott. **Oxysterols are substrates for cholesterol sulfotransferase. *J. Lipid Res.* 2007.** 48: **1343–1352.**

Supplementary key words steroid/sterol sulfotransferase • 7-ketocholesterol sulfate • cytotoxicity

In contrast to the many studies that have established the deleterious effects of oxysterols on biologic processes (1–4), there have been few studies focusing on metabolic pathways for their disposal. Of particular interest are the high levels of oxysterols in atheromas because of their association with instability and rupture, a prelude to myocardial infarction (5, 6). A major oxysterol found in atheromas as well as other tissues is 7-ketocholesterol (7-KC), which is known from cell culture studies to induce cell injury at concentrations present in vivo (7, 8); for this reason, there exists a particular focus on metabolic pathways that can lead to a reduction in its toxicity. For example, it has been shown that 7-KC is a substrate for 27-hydroxylation, thus forming a more water-soluble triol that decreases the intracellular concentration of 7-KC in macrophages (9–11). We recently reported that in addition to accelerating transport, 27-hydroxylation also prevents the loss of cell viability and in coculture nullifies the toxicity of 7-KC (12), findings consonant with the differential effects of oxysterols when used in combination (13).

Another potential metabolic pathway for the metabolism of oxysterols became apparent when it was found that one member of the SULT2 family of cytosolic sulfotransferases, SULT2B1b, has a particular affinity for cholesterol (14). Although it is well recognized that sulfonation of steroid hormones affects their biologic activity (15) and can influence their disposal (16), the concept that an analogous pathway exists for C_{27} sterols has received limited attention.

Cytosolic sulfotransferases make up a superfamily of enzymes of which the SULT2 family sulfonates steroids/sterols (17). The SULT2 family is further differentiated into two subfamilies: SULT2A1, the prototypical steroid sulfotransferase commonly referred to as dehydroepiandrosterone sulfotransferase, and SULT2B1. The *SULT2B1* gene, because of an alternative exon 1 and differential splicing, encodes two isoforms, SULT2B1a and SULT2B1b (18). Whereas human SULT2A1 and SULT2B1a avidly sulfonate the steroid pregnenolone, they do not use cholesterol effectively as a substrate; on the other hand, the SULT2B1b isozyme sulfonates cholesterol with the highest efficiency and, therefore, represents the physiologic cholesterol sulfotransferase (19).

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Abbreviations: BCD, 2-hydroxypropyl-β-cyclodextrin; CCK-8, Cell Counting Kit-8; ESI, electrospray ionization; 7-KC, 7-ketocholesterol; 7-KCS, 7-ketocholesterol sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

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In this report, we present evidence that MCF-7 cells, which highly express SULT2B1b, were significantly more resistant to the cytotoxic effect of 7-KC than 293T cells, which do not express this isozyme. Interestingly, however, using transfection of 293T cells, we were able to establish that the level of SULT2B1b expression correlated with a reduction in the toxicity level of 7-KC. In additional studies, we extended the range of C_{27} sterols that are substrates for SULT2B1b to include the 5α,6α/5β,6β-epoxy, 7α/7β-hydroxy, and 7α-hydroperoxide derivatives of cholesterol, thus expanding the potential role of this novel pathway in modulating in vivo the injurious effects of both oxysterols and hydroperoxides. Finally, we also present evidence for the first time that the sulfoconjugate of 7-KC does indeed occur in vivo, as demonstrated for human atheromatous tissue.

EXPERIMENTAL PROCEDURES

Materials

Cholesterol, 7-KC, 7α-/7β-hydroxycholesterol, and 5α,6α-/5β,6β-epoxycholesterol were purchased from Steraloids (Newport, RI). Methanol, acetonitrile, and ammonium acetate for liquid chromatography-mass spectrometry were of HPLC grade and obtained from Nacalai Tesque (Kyoto, Japan). Distilled water of HPLC grade was from Wako Pure Chemical Industries (Osaka, Japan). Cholesterol 7α-hydroperoxide was prepared from cholesterol using hematoporphyrin and visible light according to the procedure described in detail previously (20). The isotopes [^{35}S]3′-phosphoadenosine 5′-phosphosulfate (PAPS; 1.1 Ci/mmol) and [^{3}H]7-KC (40 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA) and American Radiolabeled Chemicals (St. Louis, MO), respectively.

Iodine crystals, 2-hydroxypropyl-β-cyclodextrin (BCD), PAPS, and polyethylenimine were obtained from Sigma-Aldrich (St. Louis, MO). Organic solvents were purchased from either J. T. Baker or Mallinckrodt (Phillipsburg, NJ). Silica gel TLC plates were obtained from Analtech (Newark, DE), and Immobilon-P was from Millipore (Bedford, MA). TOPO TA Cloning Kit, pcDNA3.1(+), DMEM, FBS, and Antibiotic-Antimycotic were from Invitrogen (Carlsbad, CA). PfuUltra Hotstart DNA Polymerase was purchased from Stratagene (La Jolla, CA). Oligonucleotides were purchased from Operon Biotechnologies (Huntsville, AL). Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies (Gaithersburg, MD). Goat anti-rabbit IgG conjugated to horseradish peroxidase and the LumiGLO Chemiluminescent Substrate System were obtained from KPL (Gaithersburg, MD). Proteinase inhibitor cocktail was purchased from Roche (Indianapolis, IN), and the BCA protein assay kit was from Pierce (Rockford, IL). BioMax XAR film was obtained from Kodak (Rochester, NY).

Instrumentation and analytical methods

Melting point was measured with a micro hot-stage apparatus and is uncorrected. The ^{1}H-NMR spectrum was recorded with a JEOL EX-270 spectrometer (JEOL, Tokyo, Japan) operated at 270.05 MHz. Chemical shifts are given as the δ value with tetramethylsilane as an internal standard (s, singlet; d, doublet; m, multiplet). LC-MS analysis was performed using a Finnigan LTQ linear ion-trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with an electrospray ionization (ESI) source and coupled to a Paradigm MS4 pump (Michrom Bioresources, Auburn, CA) and an autosampler (HTC PAL; CTC Analytics, Zwingen, Switzerland). The ionization conditions for verifying the structure of 7-ketocholesterol sulfate (7-KCS) were as follows: ion source voltage, –4 kV; capillary temperature, 270°C; capillary voltage, –33 V; sheath gas (nitrogen gas) flow rate, 50 arbitrary units; auxiliary gas (nitrogen gas) flow rate, 5 arbitrary units; tube lens offset voltage, –135 V. For tandem MS analysis, helium gas was used as the collision gas and the normalized collision energy was set at 25%. The LC separations were conducted on a semimicro column, TSKgel ODS-100V (5 μl, 150 × 2 mm inner diameter; Tosoh Co., Tokyo, Japan) by isocratic elution using acetonitrile-5 mM ammonium acetate buffer, pH 6.0 (3:1, v/v), as a mobile phase at a flow rate of 200 μl/min.

Synthesis of 7-KCS

To prepare the sodium salt of 7-KCS, a solution consisting of 50 mg of 7-KC in 1 ml of dry pyridine was added to freshly prepared sulfur trioxide-pyridine complex and stirred at room temperature overnight. After evaporation of the pyridine in vacuo at room temperature, the residue was redissolved in 5 ml of water and passed through a short pad of Cosmosil 140C_{18}-OPN (Nacalai Tesque) on a sintered glass filter. After washing with water, the sterol sulfate was eluted with methanol and the eluate was chromatographed on silica gel using chloroform-methanol (7:1, v/v). The yield was 100%, and the colorless solid had a melting point of 125–129°C. For ESI-MS, *m/z* 479.4 $[M\text{-}H]^-$ (100%); for ESI-MS, *m/z* 96.8 $[HSO_4]^-$. For ^{1}H-NMR ($CDCl_3$) δ: 0.683 (3H, s, H-18), 0.863 (3H, d, J = 6.48 Hz, H-26 or H-27), 0.867 (3H, d, J = 6.75 Hz, H-26 or H-27), 0.923 (3H, d, J = 6.75 Hz, H-21), 1.197 (3H, s, H-19), 4.338 (1H, m, H-3α), 5.700 (1H, s, H-6).

7-KCS formation by SULT2 enzymes

Human SULT2A1, SULT2B1a, and SULT2B1b were overexpressed in bacteria as glutathione *S*-transferase fusion proteins, cleaved, and affinity-purified as described previously (19). A 20 μl reaction volume contained 0.1 mM PAPS and purified enzyme preparation as described previously (14). Briefly, a mixture consisting of SULT2A1 (4 μg), SULT2B1a (4 μg), and SULT2B1b (1 μg) in 0.1 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$, 0.2 mM BCD, and [^{3}H]7-KC in 4% ethanol was prepared. Reactions were carried out at 37°C for 5 min and stopped at 100°C for 5 min. After adding 10 μl of 5 mg/ml cholesterol sulfate as carrier, 5 μl aliquots were applied to TLC plates. Chromatography was carried out using the solvent system chloroform-methanol-acetone-acetic acid-water (8:2:4:2:1), after which the plates were dried and exposed to iodine vapor to visualize the location of 7-KCS. The iodine-adsorbed spots were excised and placed into counting vials containing 5 ml of scintillation cocktail, and the radioactivity was determined by liquid scintillation spectrometry.

Oxysterol sulfate formation by SULT2B1b

Human SULT2B1b was overexpressed in bacteria and affinity-purified as described above. A 20 μl reaction volume contained 0.1 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM BCD, oxysterol substrate, and 5 μM [^{35}S]PAPS. The amount of SULT2B1b used for each reaction (μg/tube) was as follows: cholesterol (0.4 μg), 7-KC (1 μg), 7α-hydroxycholesterol (0.4 μg), 7β-hydroxycholesterol (1 μg), 5α,6α-epoxycholesterol (0.1 μg), and 5β,6β-epoxycholesterol (0.1 μg). Enzymatic reactions, chromatography, iodine-adsorbed staining, and liquid scintillation spectrometry counting were carried out as described above.

Construction of SULT2B1b and PAPS synthetase 1 vectors

PCR was used to isolate pcDNA-SULT2B1b. The PCR product was amplified using pGEX-6P-3-SULT2B1b (19) as a template

and the SULT2B1b-specific primers 5′-TCTAGAATGGACGGGCCCGCCGAGCCCCAGATC-3′ (sense) and 5′-GCGGCCGCTTATGAGGGTCGTGGGTG-3′ (antisense). The underlined areas indicate *Xba*I and *Not*I sites, respectively. PCR conditions with PfuUltra Hotstart DNA Polymerase were as follows: denaturing at 95°C for 2 min, followed by 25 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR product was subcloned into pCR2.1-TOPO using the TOPO TA Cloning Kit according to the manufacturer's protocol and sequenced. After digestion with *Xba*I and *Not*I, DNA was ligated to *Xba*I/*Not*I-digested pcDNA3-cMyc (kindly provided by Dr. Inohara Naohiro, Department of Pathology, University of Michigan Medical School). To isolate pcDNA-PAPS synthetase 1 (PAPSS1), after digestion of pGEX-6P-3-PAPSS1 with *Bam*HI and *Not*I, DNA was ligated to *Bam*HI/*Not*I-digested pcDNA3.1(+) (21).

Cell culture

293T cells were generously provided by Dr. Pamela Schwartzberg at the National Human Genome Research Institute (National Institutes of Health, Bethesda, MD). MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown at 37°C in DMEM supplemented with 10% (v/v) FBS and Antibiotic-Antimycotic in 5% CO_2. Media were changed every other day.

Transfection

293T cells were seeded in 6 cm Falcon dishes (Franklin Lakes, NJ) at 16.5×10^5 cells/dish on the day before being transfected. Transfections were carried out using the Calphos Mammalian Transfection Kit according to the protocol of BD Biosciences Clontech (Mountain View, CA). pcDNA-SULT2B1b plus pcDNA-PAPSS1 or empty vector as a control was mixed with 2 M $CaCl_2$ and HEPES-buffered saline and incubated for 20 min. After addition of the mixture, media were incubated for 8 h and changed to DMEM with 10% (v/v) FBS and Antibiotic-Antimycotic in 5% CO_2.

Cytotoxicity analysis

Transfected (48 h) 293T cells (10×10^4) were seeded using DMEM without phenol red on 24-well plates coated with 0.05% polyethylenimine and used to assay for sterol cytotoxicity, which was performed according to the manufacturer's protocol (Dojindo Molecular Technologies). Either 7-KC or 7-KCS dissolved in 45% (w/v) BCD was added to culture media, and the cells were incubated at 37°C in 5% CO_2 for 24 h. After this time period, 20 μl of CCK-8 was added to the cultures and incubations were continued for an additional 1.5 h. Reactions were stopped by the addition of 50 μl of 1% (w/v) sodium dodecyl sulfate solution. Care was taken to avoid light exposure. Absorbance was measured at 450 nm using the μQuant plate reader (BioTek, Winooski, VT). Data were statistically analyzed by two-way ANOVA using GraphPad Prism (San Diego, CA).

HPLC analysis after transcription

293T cells were transfected with pcDNA-SULT2B1b and pcDNA-PAPSS1 or pcDNA3.1 as a control for 48 h as described previously and then reseeded in a 10 cm dish at 1×10^5 cells/dish. [^{3}H]7-KCS (5 nM) in DMEM containing 30 μM BCD and 10% delipidated FBS (Monobind, Lake Forest, CA) and Antibiotic-Antimycotic was added to the cell culture and incubated for 48 h at 37°C in 5% CO_2. The medium was extracted as described above and analyzed for 7-KCS formation. A model 1100 Hewlett-Packard instrument was used with a photoarray detector set at 210 and 225 nm. A C18 reverse-phase 250 × 4.6 mm chromatograph with a 4 μ silica column (catalog number OOG-4375-EO; Phenomenex, Torrance, CA) was used with a binary system of methanol-water beginning at 30% methanol and increasing to 100% methanol over a 30 min period and then continuing isocratically for an additional 30 min. The flow rate was constant at 0.8 ml/min. Samples were counted at constant efficiency using liquid scintillation spectrometry (Beckman).

Western blotting

Transfected cells were washed two times with PBS and disrupted using a plastic cell scraper and cold RIPA lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100) with proteinase inhibitor cocktail. After centrifugation for 20 min, the supernatant was collected and the protein concentration was determined using the BCA protein assay kit. Samples (40 μg) were subjected to electrophoresis on a NuPage 4–12% Bis-Tris gel (Invitrogen) using MOPS/SDS running buffer and transferred to a polyvinylidene fluoride membrane (Immobilon-P). Membranes were soaked in a solution of 5% dry milk in TBS containing 0.05% Tween 20 (T-TBS) for 30 min with gentle shaking. Membranes were then exposed to either SULT2B1b (1:2,000; HL4360 #9) or PAPSS1 (1:2,000; HL4004) overnight at 4°C and washed three times with T-TBS for 5 min. Goat anti-rabbit IgG conjugated to horseradish peroxidase (1:55,000) was added and incubated for 1 h at room temperature. After washing three times with T-TBS for 5 min, LumiGLO chemiluminescent substrate was used according to the manufacturer's protocol before exposing membranes to X-ray film for 1 min.

Measurement of cellular cholesterol content

293T cells were transfected as described previously. After quantifying the cell number, cells were extracted with chloroform/1% Triton X-100 and the organic phase was collected. The chloroform was removed by heat block and the use of the Savant SpeedVac system (GMI, Ramsey, MN). The dried cholesterol content was measured using the Cholesterol/Cholesteryl Ester Quantitation Kit according to the manufacturer's instructions (BioVision, Mountain View, CA) and expressed as μg/10^6 cells.

Analysis of human atheroma

Fresh-frozen specimens of human atheroma, obtained from autopsies under an approved protocol, were generously supplied by Dr. Allen Burke at the CVPath Institute (Gaithersburg, MD). Approximately 500 mg sections were added to 9.5 ml of methanol, and using a microtip probe (Sonicator 3000; Misonix, Farmingdale, NY), the samples were sonicated (2.5 W) at intervals for a total of 3 min with temperature monitoring and cooling in ice water so that the temperature did not increase to >60°C. The specimens were then centrifuged, and the supernatant was decanted and taken to dryness. The residue was redissolved in 0.5 ml of methanol and centrifuged, and the supernatant was used for HPLC analysis.

Reverse-phase HPLC was carried out using a Hewlett-Packard 1100 instrument and a 150 × 4.6 mm C18 column (Aqua 3 μ, 125 A; Phenomenex) that was maintained at 60°C. A gradient of methanol-water was used beginning at 45% methanol and increased to 100% methanol over a 40 min period with a constant flow rate of 0.8 ml/min. Under these conditions, it was determined that standards of 7-KCS and 7-KC had retention times of 26.5 and 34 min, respectively, using a multi-wavelength detector (210, 225, and 233 nm). Methanolic aliquots of the atheroma specimens were injected onto the column, and fractions were collected at 2 min intervals for 48 min. Fractions obtained from 26 to 28 min were pooled for each specimen and taken to

dryness. The pooled and lyophilized HPLC samples were then dissolved in 50 μl of 75% acetonitrile, and 10 μl aliquots were used for LC-ESI-MS analysis.

RESULTS

Enzyme catalysis

That SULT2B1b has a penchant for C_{27} sterols, in contrast to the SULT2A1 and SULT2B1a isozymes, is demonstrated in **Fig. 1**. SULT2B1b is 1 order of magnitude more active in sulfonating 7-KC than the other two steroid sulfotransferases (**Table 1**). The ability of SULT2B1b to sulfonate C_{27} sterols in addition to cholesterol and 7-KC was extended to include the α- and β-isomers of 7-hydroxycholesterol and 5,6-epoxycholesterol (**Table 2**). Interestingly, although SULT2B1b sulfonated cholesterol with the highest efficiency, its efficiency in sulfonating 7α-hydroxycholesterol was nearly as good (Table 2). Additionally, SULT2B1b was able to sulfonate the 7α-hydroperoxide derivative of cholesterol, although not as effectively as cholesterol, 7α-hydroxycholesterol, and 7-KC (**Table 3**). By contrast, the rate of dehydroepiandrosterone sulfate formation by SULT2B1b was <10% of that occurring with the prototypical steroid sulfotransferase, SULT2A1 (data not shown).

Cytotoxicity studies

For the initial studies, the 293T cell line was chosen because it does not normally express SULT2B1b; furthermore, 293T cells do not express PAPS synthetase, which produces the universal sulfonate donor molecule, PAPS. Therefore, because PAPS is essential for sulfonation to occur, 293T cells were cotransfected with a cDNA for PAPSS1 as well as a cDNA for SULT2B1b.

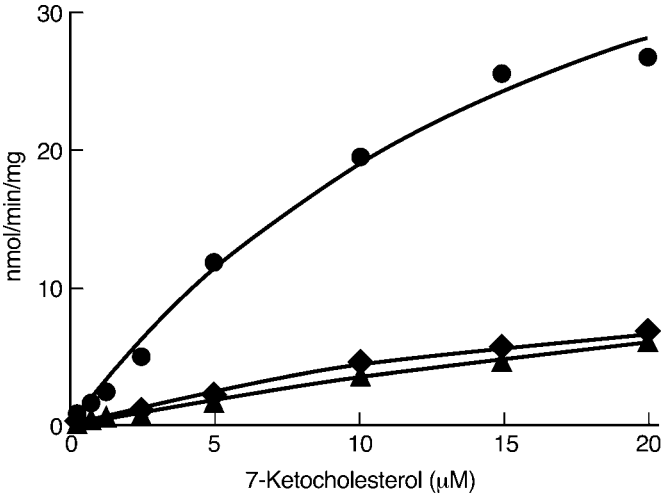

Fig. 1. Kinetic analysis of human SULT2A1 (triangles), SULT2B1a (diamonds), and SULT2B1b (circles) using 7-ketocholesterol (7-KC) as substrate. Proteins were overexpressed in bacteria as glutathione *S*-transferase fusion proteins, cleaved, and affinity-purified. Sulfotransferase assays were carried out as described in Experimental Procedures.

TABLE 1. SULT2 enzyme 7-KC kinetic values

Enzyme	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	M	$nmol/min/mg$	s^{-1}	$M^{-1}\ s^{-1}$
SULT2B1b	7.7×10^{-6}	29.1	2.0×10^{-2}	2.6×10^{3}
SULT2B1a	2.4×10^{-5}	6.7	4.2×10^{-3}	1.8×10^{2}
SULT2A1	1.7×10^{-5}	7.0	3.9×10^{-3}	2.3×10^{2}

7-KC, 7-ketocholesterol. Human SULT2 enzymes were overexpressed as fusion proteins, cleaved, and affinity-purified. SULT2A1 (4 μg), SULT2B1a (4 μg), and SULT2B1b (1 μg) were placed in 0.1 mM Tris-HCl (pH 7.5) containing 0.1 mM 3′-phosphoadenosine 5′-phosphosulfate (PAPS), 5 mM $MgCl_2$, 0.2 mM 2-hydroxypropyl-β-cyclodextrin (BCD), and [^{3}H]7-KC. Reactions were carried out at 37°C for 5 min and stopped at 100°C for 5 min, and reactants were analyzed as described in Experimental Procedures.

As shown in **Fig. 2**, although adding an increasing amount of 7-KC to the culture medium caused a progressive loss in cell viability with and without transfection, the transfected cells nevertheless demonstrated a significant reduction in cell injury at all concentrations of 7-KC used except the very highest concentration, at which a protective effect was not found (Fig. 2). To ascertain whether a significant alteration in the content of cell cholesterol might contribute to the cytotoxic effect of 7-KC, cholesterol was measured before and after transfection; the results revealed no significant difference [28.6 ± 11.4 $\mu g/10^6$ cells (n = 5) for mock-transfected cells vs. 28.3 ± 8.8 $\mu g/10^6$ cells (n = 5) for the transfected cells].

That protection from loss of cell viability with transfected 293T cells was associated with the formation of SULT2B1b and the production of 7-KCS is supported by the results of Western blotting and HPLC analysis. That is, there was virtually no 7-KCS found in the medium of mock-transfected cells, in contrast to the >55% conversion with the transfected cells (**Fig. 3**). Correspondingly, there was a striking increase in the content of SULT2B1b protein after transfection of 293T cells with the SULT2B1b cDNA, as shown by Western analysis (**Fig. 4**). Furthermore, as these were cotransfection experiments, there was also a marked increase in the expression of PAPSS1, as shown by immunoblotting (Fig. 4). Importantly, it was found that 7-KCS (as well as cholesterol), when added to the culture medium of 293T cells in amounts equimolar to 7-KC, did not cause any loss of cell viability (**Fig. 5**). Additional support for the protective effect of sulfonation of 7-KC was obtained when the cytotoxicity of 7-KC was tested with MCF-7 cells and compared with 7-KC cytotoxicity with 293T cells. MCF-7 cells, which in contrast to the nonexpressing 293T cells highly express SULT2B1b, were significantly and impressively more resistant to the cytotoxic effect of 7-KC than 293T cells (**Fig. 6**). Of note was the finding that, in the case of MCF-7 cells, there was an apparent increase in cell survival at the two lowest concentrations of 7-KC used, followed by a progressive decline in cell survival as the dose of 7-KC was increased further (Fig. 6). This paradox can be explained, in part, by the fact that BCD (3 mM) by itself (as in the zero 7-KC control cells) was somewhat toxic to the MCF-7 cells, in contrast to 293T cells, in which this phenomenon was not seen (Figs. 2, 6). This mild degree of

TABLE 2. SULT2B1b substrate kinetic values

Substrate	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	M	*nmol/min/mg*	s^{-1}	$M^{-1}\ s^{-1}$
Cholesterol	1.1×10^{-6}	25.2	1.7×10^{-2}	1.6×10^{4}
7α-Hydroxycholesterol	2.3×10^{-6}	50.3	3.5×10^{-2}	1.5×10^{4}
5α,6α-Epoxycholesterol	2.5×10^{-6}	19.7	1.4×10^{-2}	5.4×10^{3}
7-KC	5.5×10^{-6}	38.2	2.6×10^{-2}	4.8×10^{3}
7β-Hydroxycholesterol	5.9×10^{-6}	36.2	2.5×10^{-2}	4.2×10^{3}
5β,6β-Epoxycholesterol	3.9×10^{-6}	22.6	1.6×10^{-2}	4.0×10^{3}

The reaction mixture (20 μl) consisted of 0.1 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM BCD, substrate, and 5 μM [^{35}S]PAPS. The optimal amount of human SULT2B1b, which was overexpressed as a fusion protein, cleaved, and affinity-purified, was determined for each substrate as follows: cholesterol (0.4 μg), 7-KC (1 μg), 7α-hydroxycholesterol (0.4 μg), 7β-hydroxycholesterol (1 μg), 5α,6α-epoxycholesterol (0.1 μg), and 5β,6β-epoxycholesterol (0.1 μg). Reactions were carried out at 37°C for 5 min and stopped at 100°C for 5 min, and the sulfonated products were analyzed as described in Experimental Procedures.

toxicity (presumably attributable to the extraction of cellular cholesterol by BCD) could be overcome by the addition of cholesterol (20 μM) to the zero 7-KC control cells (data not shown). This fact notwithstanding, however, using as a zero 7-KC control, BCD plus cholesterol did not completely eliminate the apparent increase in cell survival at the two lowest doses of 7-KC tested (data not shown).

Detection of 7-KCS in atheromatous tissue

Figure 7 shows mass chromatographic monitoring of the deprotonated ion $[M\text{-}H]^-$ at *m/z* 479.3 and the fragmented ion $[HSO_4]^-$ at *m/z* 96.8 as well as the collision-induced dissociation spectra for authentic 7-KCS and fractions 26–28 from HPLC analysis of two atheroma specimens. Retention time and collision-induced dissociation spectra of both atheroma samples were completely identical to those of standard 7-KCS. Although a definitive quantification is planned, there was no formal attempt at quantifying the amount of 7-KCS in this study. It should be noted that 7-KCS is very soluble in methanol, which was the solvent used for extraction, suggesting that recovery of 7-KCS should have been fairly efficient. Nevertheless, at this time we can only make estimates, and based on the area obtained for a 100 ng standard, we estimate the amount of 7-KCS in one specimen at ~150 ng, whereas for the other specimen the estimate is ~5,000 ng.

TABLE 3. SULT2B1b sulfonation of C_{27} oxysterols

Sterol Substrate	Enzyme Activity
	nmol/min/mg
Cholesterol	7.5
7-KC	7.1
7α-Hydroxycholesterol	21.0
7β-Hydroxycholesterol	1.9
7α-Hydroperoxide	4.8

Reactions were carried out for 20 min at 37°C in a solution of 0.08 M Tris (pH 7.1) containing 87 M [^{35}S]PAPS, 4 mM $MgCl_2$, and 1.3 mM BCD. Substrate and protein concentrations were 20 M and 10 g/ml, respectively. [^{35}S]sterol sulfate formation was analyzed after extraction with methylene blue (14).

DISCUSSION

In this study, we focused on the important role that expression of SULT2B1b has in modifying the cytotoxic effect of 7-KC; presumably, a similar effect would be seen with other toxic oxysterols. We previously demonstrated that oxysterols generated by side chain oxidation, such as 27-hydroxycholesterol, are substrates for SULT2B1b sulfonation at the C_3 position (14). In the current studies, we found that ring-modified structures, such as the naturally occurring 7α-hydroxy and 7α-hydroperoxide metabolites of cholesterol, can also serve as substrates for SULT2B1b, indicating a major role for this enzyme in the metabolism of a class of compounds considered to be an important cause of cell injury. The hydroperoxide is of particular interest because it is considered a photo-oxidation prod-

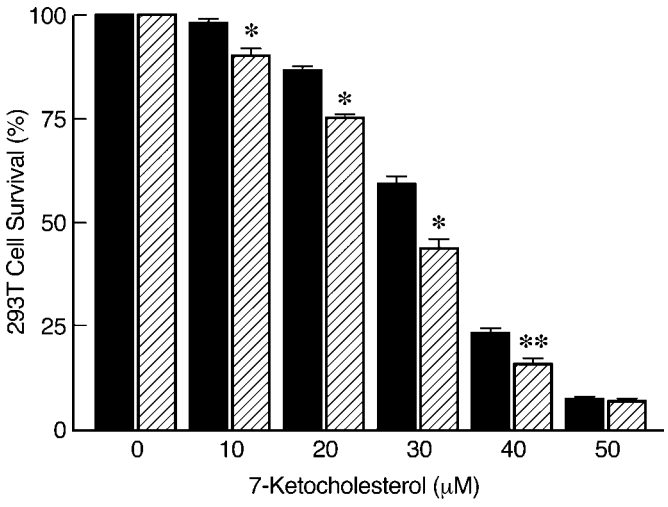

Fig. 2. Cytotoxicity analysis of 293T cells after exposure to 7-KC. Cells were either mock-transfected (hatched columns) or cotransfected with SULT2B1b and 3′-phosphoadenosine 5′-phosphosulfate synthase 1 (PAPSS1) (black columns) as described in Experimental Procedures. After transfection (48 h), cells were seeded to 24-well plates and incubated with 7-KC for 24 h. Cytotoxicity was determined by Cell Counting Kit-8 (CCK-8) analysis as described in Experimental Procedures. Data (means ± SD; n = 4) are presented as percentage survival compared with untreated control cells. * $P < 0.001$, ** $P < 0.01$.

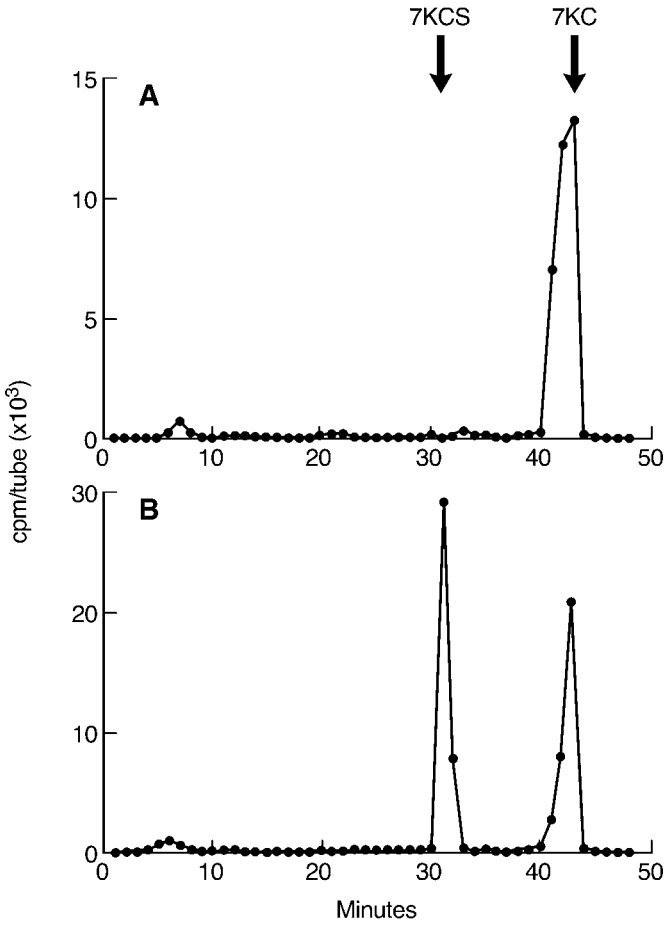

Fig. 3. HPLC analysis after transfection of 293T cells. Transfections were carried out, cells were incubated with [^{3}H]7-KC for 48 h, and the medium was analyzed by HPLC as described in Experimental Procedures. Fractions were collected at 2 min intervals. A: Mock transfections. B: SULT2B1b and PAPSS1 cotransfections. 7-KCS, 7-ketocholesterol sulfate.

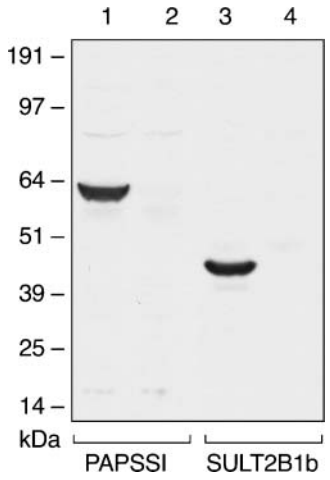

Fig. 4. Western analysis of transfected 293T cells. Cells were either mock-transfected (lanes 2 and 4) or cotransfected with PAPSS1 and SULT2B1b (lanes 1 and 3) as described in Experimental Procedures. Soluble cell extracts were prepared, and immunoblotting (40 μg/lane) was carried out as described in Experimental Procedures.

uct generated in the retina (22, 23), is lethal when added to cell cultures, and functions as a precursor of other toxic oxysterols (24).

Oxysterols are present in tissues with a large excess of cholesterol, creating a ratio of cholesterol to oxysterol that ranges from 10^3 to 10^6 (25). Such a large concentration disparity might be expected to reduce the effectiveness of SULT2B1b in being able to sulfonate 7-KC added to culture medium at only 5 nM, which when taken up by the 293T and MCF-7 cells would have to compete with the large excess of endogenous cholesterol; however, this turned out not to be the case. A plausible explanation for this is that cellular cholesterol is virtually all confined to membranes and any cytosolic cholesterol would be mostly esterified and thus unavailable to be acted on by SULT2B1b. Thus, the magnitude of cholesterol competition would appear to be more apparent than real. In addition, it is known that there are many different specific lipid carriers, some identified and some with "orphan" status, that regulate access to enzymes rather than a pot of molecules floating around in a cytosolic soup (26). Perhaps a good example is the relatively small amount of 7α-hydroxylated intermediates in bile acid synthesis that are 27-hydroxylated by an enzyme for which cholesterol is also a substrate.

Other than the metabolism of 7α-hydroxycholesterol and 27-hydroxycholesterol, relatively little is known about the metabolic fate of other oxysterols. The key intermediate in bile acid synthesis by the classical or neutral pathway, 7α-hydroxycholesterol, is generated from cholesterol by the microsomal enzyme CYP7A1, an enzyme that is expressed only in the liver, where 7α-hydroxycholesterol is further metabolized to the primary bile acid products chenodeoxycholic and cholic acids (27). On the other hand, 7α-hydroxylation of other sterols can occur in tissues other than the liver, steps in oxysterol metabolism that can also lead to bile acid synthesis by an alternative pathway carried out by the microsomal enzymes CYP39A1 (24-hydroxycholesterol) and CYP7B1 (25- and 27-hydroxycholesterol) (27). The key intermediate in bile acid synthesis by the alternative or acidic pathway is 27-hydroxycholestesrol (28), which is produced from cholesterol by the mitochondrial enzyme CYP27A1, an enzyme that is expressed in most tissues (9, 29, 30). Of some interest was the finding that cholesterol sulfate (produced by SULT2B1b) can be 27-hydroxylated by CYP27A1 at a higher rate than cholesterol itself (31).

The major oxysterol in human atherosclerotic plaques is 27-hydroxycholesterol, with the second most abundant being 7-KC (32). Likewise, the major circulating oxysterol in normal human plasma is 27-hydroxycholesterol, with 7-KC being present to a lesser degree (33, 34). Although two metabolic pathways exist for the metabolism of 27-hydroxycholesterol, only naturally occurring primary bile acids have been identified as metabolites in humans (28). Nevertheless, the 3-sulfate derivative of 27-hydroxy-

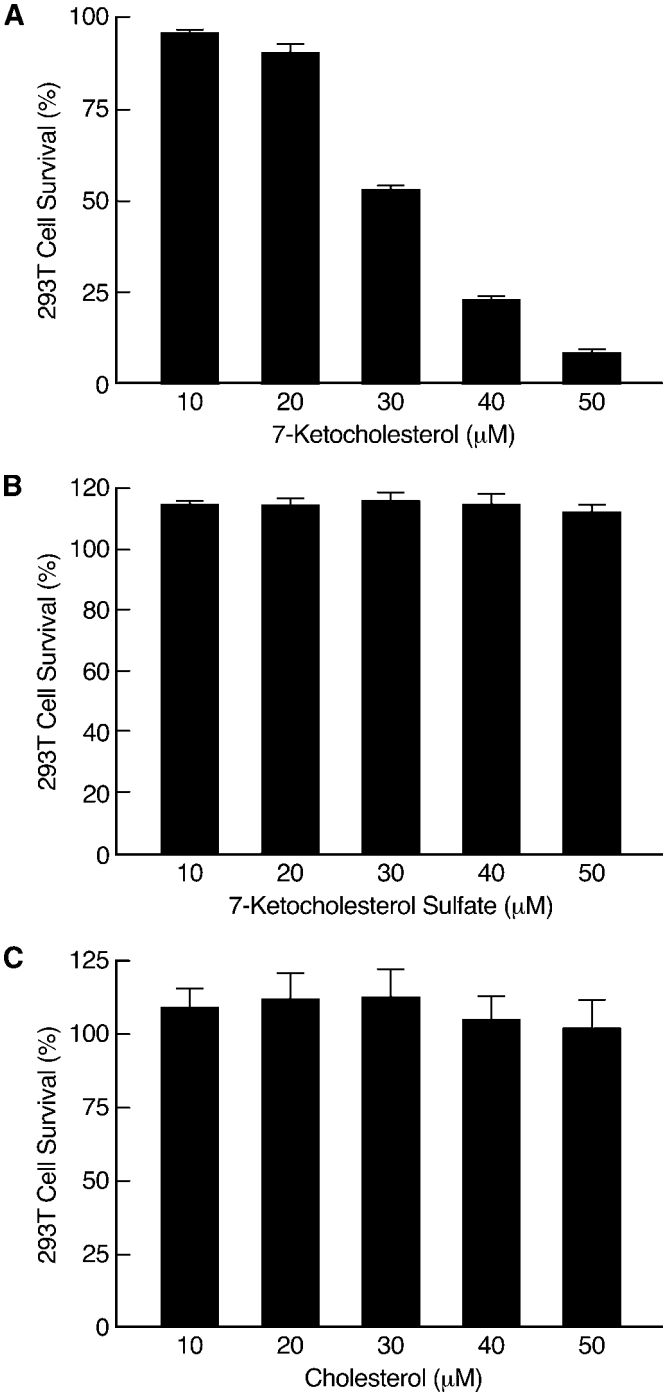

Fig. 5. Cytotoxicity analysis. After 24 h of incubation of 293T cells with 7-KC (A), 7-KCS (B), and cholesterol (C), cell survival was determined by CCK-8 analysis as described in Experimental Procedures. Data represent means ± SD (n = 4).

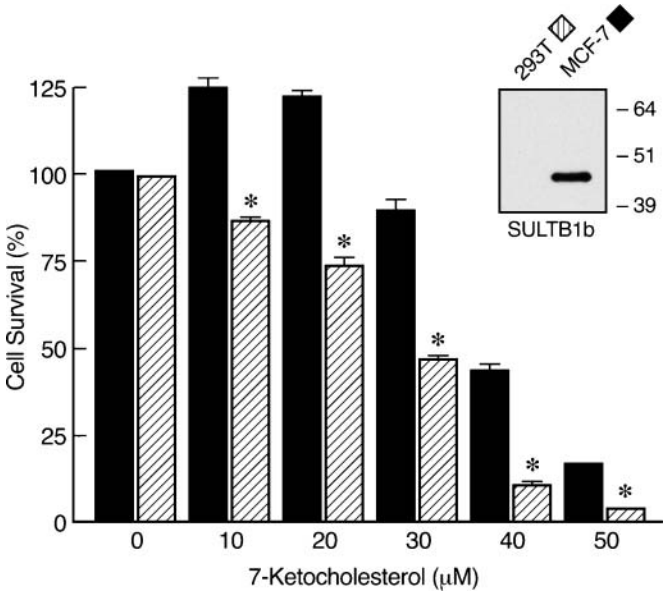

Fig. 6. Cytotoxicity analysis. After 24 h of incubation of either 293T (hatched columns) or MCF-7 (black columns) cells with 7-KC, cell survival was determined by CCK-8 analysis as described in Experimental Procedures. Data represent means ± SD (n = 4). * $P < 0.001$. The inset shows Western analysis of SULT2B1b expression by 293T and MCF-7 cells.

cholesterol is a major oxysterol component in feces from normal human infants (35), and 27-hydroxycholesterol is a substrate for SULT2B1b, implying that 27-hydroxycholesterol-3-sulfate can be produced in tissues that express this enzyme (14). It is of interest that 24*S*-hydroxycholesterol, an oxysterol generated only in the central nervous system, has been found to circulate in the plasma of children with cholestasis as the 3-sulfate-24-glucuronide (36), thus following a metabolic pathway more characteristic of steroid hormones. Furthermore, a patient with Niemann-Pick disease excreted abnormal amounts of unusual bile acids in urine that were sulfated at the C_3 position; these acids were shown to have a 3β-hydroxy-Δ^{5-6} structure and to carry an oxygen function at the C_7 position. Additionally, high levels of 25-hydroxycholesterol-3-sulfate have been identified in normal human hepatic nuclei and mitochondria (37). With regard to the latter observation, it should be noted that SULT2B1b has been localized in nuclei of human placenta by differential centrifugation and immunoblotting (38) as well as in nuclei of THP-1 macrophages by confocal microscopy (our unpublished data). The point to be made here is that, although 7-KCS had not been identified previously in human tissues, its formation was highly likely to occur. Now, based on this report, the in vivo formation of 7-KCS has been determined definitively.

Considering the potential importance of 7-KC as an autoxidation product that causes cell injury, it is surprising that relatively little is known about its metabolism in vivo. In early animal studies involving rats, 7-KC was converted into unusual bile acids (i.e., unidentified polar metabolites) (39, 40). Studies that focused on the relationship of the metabolism of 7-KC and cholesterol were carried out in rats (41) and mice (42) with essentially the same results. In contrast to cholesterol, which was retained in the liver, 7-KC was rapidly excreted into the intestine and urine as unidentified water-soluble metabolites. Distinctly different pathways thus metabolize cholesterol and 7-KC. All of the studies of 7-KC metabolism dem-

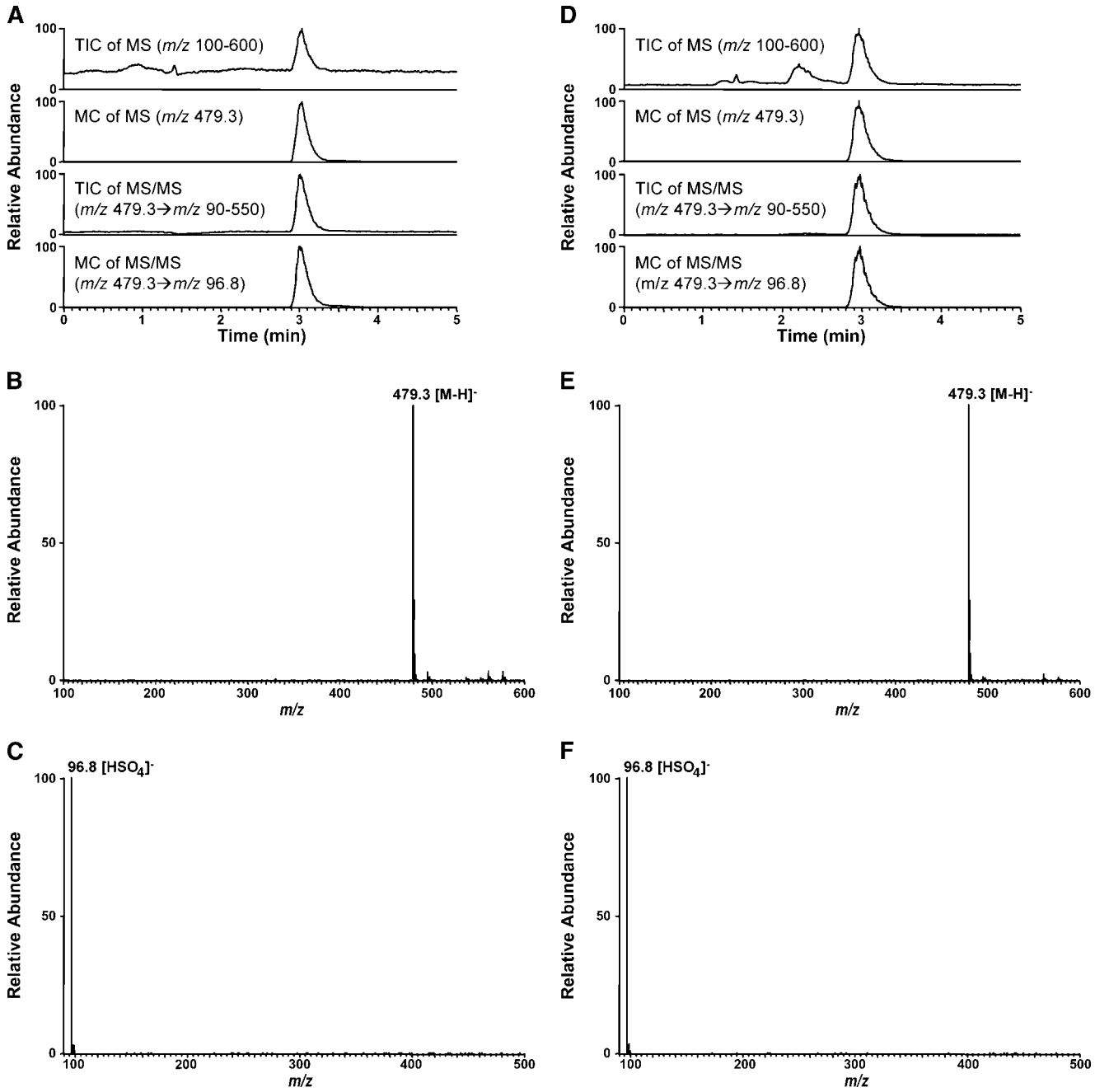

Fig. 7. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis of 7-KCS of human atheromas. Extraction of 7-KCS from atheromatous tissue was carried out, and the extracts were analyzed first by HPLC followed by LC-ESI-MS/MS as described in Experimental Procedures. A–C: Authentic 7-KCS. D–F: Extracts of human atheromas. A, D: Total ion chromatograms (TIC) in negative ion mode monitored with *m/z* 100–600 (top section), mass chromatograms (MC) in negative ion mode monitored with *m/z* 479.3 of $[M\text{-}H]^-$ (second section), total ion chromatograms of product ion scan monitored with *m/z* 90–550 (third section), and mass chromatograms of product ion scan monitored with *m/z* 96.8 of $[HSO_4]^-$ (bottom section). B, E: Negative ion mass spectra of the peaks eluted at 3.0 min. C, F: Collision-induced dissociation spectra obtained for the $[M\text{-}H]^-$ ion of 7-KCS.

onstrated that some of the administered radioactive material was reduced in the liver to 7β-hydroxycholesterol but not to 7α-hydroxycholesterol.

Previous in vitro studies established that 7-KC is a substrate for CYP27A1 (12, 43), and our studies established that it is also a substrate for SULT2B1b. Because we showed previously that 27-hydroxycholesterol is also a substrate for SULT2B1b, it is reasonable to speculate that 27-hydroxy-7-ketocholesterol-3-sulfate may occur in vivo. Together, the findings of the in vitro studies provide new insights regarding the possible identities of these biologically active oxysterols.

As reported here for the first time, 7-KCS is clearly formed in vivo as demonstrated for human atheromatous

tissues using LC-ESI-MS analysis, a technique commonly used to identify a variety of biologically important substances. This is an important finding because, as stated previously, high levels of oxysterols found in atheromas can lead to instability and rupture, a prelude to myocardial infarction (5, 6). A major oxysterol found in atheromas as well as other tissues is 7-KC, which is known from cell culture studies to induce cell injury at concentrations present in vivo (7, 8); however, as we report here, the sulfoconjugate of 7-KC is completely nontoxic to cells that are sensitive to the adverse effects of 7-KC. Thus, SULT2B1b, which is expressed in human macrophages (our unpublished data), offers an important in vivo metabolic route for the efficient detoxification of 7-KC as well as other cytotoxic oxysterols.

Finally, it should be noted that as reported recently, SULT2B1b can also play an important role in oxysterol regulation of cell signaling by the liver X receptor (44). Liver X receptor, which is activated by oxysterols (45), is involved in the regulation of the expression of genes engaged in fatty acid and cholesterol metabolism (46). It now appears that oxysterols have a broad range of biological effects and that SULT2B1b plays a significant role throughout the spectrum. Thus, not only does SULT2B1b inactivate classes of oxysterols that are cytotoxic, it also inactivates classes of oxysterols involved in cell signaling.

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