Short Communication

Sulfotransferase 4A1 Increases Its Expression in Mouse Neurons as They Mature[□]

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ABSTRACT

Cytosolic sulfotransferases (SULTs) catalyze sulfation and play essential roles in detoxification of xenobiotics as well as inactivation of endobiotics. SULT4A1, which was originally isolated as a brain-specific sulfotransferase, is the most highly conserved isoform among SULTs in vertebrates. Here, expression of SULT4A1 was examined in neuron-enriched and neuron-glia mixed cells derived from mouse embryo brains at day 14 gestation and mixed glia from 2-day-old neonate brains. Western blots showed an increase of SULT4A1 expression as neurons matured. Reverse-transcription polymerase chain reaction and agarose

gel analysis found two different forms (variant and wild type) of SULT4A1 mRNA in neurons; the level of wild type correlated with the protein level of SULT4A1. SULT1E1 was not expressed in mouse brains, neuron-enriched cells, or mixed glia cells. SULT1A1 protein was only detected in adult brains. Immunofluorescence staining of neuron-glia mixed cells confirmed selective expression of SULT4A1 in neurons, including dopaminergic neurons, but not in either astrocytes or microglia. Thus, SULT4A1 is a neuron-specific sulfotransferase and may play a role in neuronal development.

Introduction

Cytosolic sulfotransferases (SULTs) are enzymes which catalyze sulfation by transferring the sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate to substrate (Gamage et al., 2006). The total 15 human and 22 mouse SULTs are divided into four and six families in humans and mice, respectively, ubiquitously expressed in various tissues and organs and collectively sulfate numerous xenobiotics and endobiotics. Characteristically, SULT1 enzymes sulfate a simple phenol group, such as *p*-nitrophenol, whereas the SULT2 enzymes are known to sulfate steroid hormones, such as dehydroepiandrosterone (DHEA), estrogen, and bile acids (Wang and James, 2005; Gamage et al., 2006). An exception to these characteristics, SULT1E1 (the only member within the SULT1E subfamily and called estrogen sulfotransferase) sulfates 17 β -estradiol (E2) with high affinity at nanomolar concentrations of K_m values (Falany et al., 1995).

Contrary to the other ubiquitous expression, SULT4A1 (the only member within the SULT4 family) is only expressed in brains. Although cDNAs were cloned from human and rodent brains more than 15 years ago (Falany et al., 2000; Sakakibara et al., 2002), neither endogenous nor exogenous substrates have yet been identified. The amino acid sequence of SULT4A1 is highly conserved from mice to humans. The cross-species conservation and brain-specific expression suggest yet-unknown

physiologic functions. In fact, SULT4A1 was associated with susceptibility of schizophrenia in humans (Lewis and Minchin, 2009). Recently generated SULT4A1 knockout mice developed severe and progressive neurologic symptoms, such as tremor, rigidity, and seizure, and died postnatally at days 21–25 (Garcia et al., 2018). Here, we used mouse brains, mouse primary neurons, and glia cells for Western blot, real-time polymerase chain reaction (PCR), and immunofluorescence staining analyses to examine expression of SULT4A1, and reported that SULT4A1 is expressed in neurons, including dopaminergic neurons, but not in glia cells. Based on our finding, we discuss possible roles of SULT4A1 in the brain.

Materials and Methods

Materials. Anti-neural nuclei (anti-NeuN) antibody (ABN78) was obtained from Millipore (Billerica, MA). SULT1A1 (10911-2-AP), SULT1E1 (12522-1-AP), and SULT2B1 polyclonal antibody (12879-1-AP) were purchased from Proteintech (Rosemont, IL); SULT4A1 mouse monoclonal antibody (sc-374545) was from Santa Cruz Biotechnology (Santa Cruz, CA); ionized calcium binding adaptor molecule 1 (IBA-1) antibody (GTX100042) was from GeneTex (Irvine, CA); Alexa Fluor 488 goat anti-mouse IgG antibody and Alexa Fluor 594 goat anti-rabbit IgG antibody were from Life Technologies (Carlsbad, CA); rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody was from Chemicon (Temecula, CA); mounting medium with 4',6-diamidino-2-phenylindole and Mouse on Mouse (M.O.M.) Blocking Reagent were from Vector Laboratories (Burlingame, CA); polyclonal rabbit anti-gial fibrillary acidic protein (Z0334) was from Dako Cytomation (Carpinteria, CA); and TaqMan gene expression assays (probe and primer sets) for SULT4A1 (assay ID: Mm00489166_m1) (Fluorescein [FAM]), SULT2B1 (assay ID: Mm00450550_m1) (FAM), and mouse glyceraldehyde-3-phosphate dehydrogenase (FAM) were from Applied Biosystems (Foster, CA). All other chemicals were of the highest grade commercially available.

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ABBREVIATIONS: CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; DHEA, dehydroepiandrosterone; E14, embryonic day 14; FAM, Fluorescein; GW4064, 3-(2,6-Dichlorophenyl)-4-(3'-carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole; IBA-1, ionized calcium binding adaptor molecule 1; NeuN, neural nuclei; NIEHS, National Institute of Environmental Health Sciences; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SULT, cytosolic sulfotransferase; TH, tyrosine hydroxylase.

Animals and Cell Cultures. C57BL/6 mice were maintained under the standard condition at the National Institute of Environmental Health Sciences (NIEHS), and animal experiments were conducted according to protocols approved by the animal ethics committee at NIEHS/National Institutes of Health. The primary neuron-glia mixtures, neuron-enriched cells, and mixed glia cells were prepared according to a previous study (Chen et al., 2013). Brains were isolated from 8-week-old male mice and 2-day-old pups for subsequent Western blot analysis.

Western Blotting. The brain-derived cells and tissues were homogenized in the lysis buffer (8 M urea/1% SDS in Tris-buffered saline/Tween 20). The lysate was centrifuged and the supernatant was collected. Five micrograms of proteins was electrophoresed on a 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes at 10 V for 1 hour. The membranes were probed with a given antibody overnight at 4°C, reacted with horseradish peroxidase-conjugated secondary antibody using the WesternBright Sirius kit (Advansta, Menlo Park, CA), and visualized by a C-DiGit Blot Scanner (LI-COR, Inc., Lincoln, NE). As a loading control, β -actin levels were detected using anti- β -actin antibody (sc-47778) from Santa Cruz Biotechnology.

Immunocytochemistry. Neuron-glia mixed cells were prepared on glass bottom dishes as mentioned earlier. The cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), rinsed, and blocked with M.O.M. mouse IgG blocking reagent. The cells were subsequently blocked with reagent containing 0.4% Triton X-100/PBS, 1% bovine serum albumin, and 4% normal goat serum and washed. The primary antibody against SULT4A1, NeuN, IBA-1, glial fibrillary acidic protein, or TH was added onto the cells. After overnight reaction at 4°C, another primary antibody was added after PBS wash, followed by incubation at 4°C overnight. Alexa Fluor 488 goat anti-mouse IgG antibody and Alexa Fluor 594 goat anti-rabbit IgG antibody were reacted for 2 hours at room temperature. The mounting reagent containing 4',6-diamidino-2-phenylindole for nuclei staining was added and observed with a Zeiss LSM 710 inverted confocal microscope (Carl Zeiss Microscopy, Thornwood, NY).

Real-Time PCR (Quantitative PCR) and Reverse-Transcription PCR. Total RNA was extracted from mouse brain tissues and cells using TRIzol reagent, with which cDNAs were synthesized using a High Capacity cDNA Archive kit (Applied Biosystems). Real-time PCR was conducted using a TaqMan Universal PCR Master mix and TaqMan probes with a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers for SULT4A1 wild type, SULT4A1 variant, and β -actin were designed and amplified according to a previous report (Sidharthan et al., 2014). The products were then separated by electrophoresis on 2.0% agarose gels, stained with ethidium bromide, and visualized by UV illumination.

Statistical Analysis. Multiple groups were analyzed by one-way analysis of variance followed by Tukey's multiple comparison test. The statistical analysis was conducted using GraphPad Prism 7.04 software (GraphPad Software, San Diego, CA).

Results

SULT4A1 Expression in Mouse Brains. Extracts were prepared from brains of 8-week-old adult mice and 2-day-old neonates for subsequent Western blot analysis. Primary brain cell cultures were prepared from the mesencephalic tissues of embryonic day 14 (E14) embryos, from which neurons were enriched to about 96% purity and cultured by using an earlier described protocol (Chen et al., 2013). Mixed glia cell cultures mainly containing 70% astroglia and 30% microglia were prepared from cerebral hemispheres and midbrains of 2-day-old pups (Chen et al., 2013). In Fig. 1A, Western blot analysis showed higher expression of SULT4A1 in adults than in neonate brains. SULT4A1 was also expressed in neuron-enriched cells but not mixed glia cells. SULT1A1 was detected in only adult brains. No SULT1E1 protein was detected with any of these brains and cells. Real-time PCR was performed with RNAs to examine levels of SULT4A1 mRNA, which correlated with the protein levels in each brain and cells, except that a slight disconnect was noted between protein and mRNA levels in brains (Fig. 1B). SULT4A1 mRNA was also present in neonate brains and neuron-enriched cells in addition to adult brains. As for SULT1E1, no mRNA was detected (data not shown). These observations indicate that primary neurons express only SULT4A1 at protein levels. It is also noted that none of these SULTs are expressed in glia cells.

Fig. 1. Neuron-specific expression of SULT4A1. (A) Western blots. SULT expressions were examined in 8-week-old adult mouse brains, 2-day-old neonate brains, mixed glia cells, and neuron-enriched cells using an anti-SULT4A1, SULT1A1, or SULT1E1 antibody. Sample preparations were independently conducted three times, and the representative Western blot image was indicated. (B) Real-time PCR. SULT4A1 mRNA levels using the tissues or cells as described in (A). Each value is shown as the mean $\pm$ S.D. ($n = 3$). Asterisks indicate significant differences between experimental groups ($*P < 0.05$).

SULT4A1 Expression During Neuron Maturation. Primary mid-brain neuron-glia cells were prepared from E14 embryos. During the 7-day culturing period, neurons mature by extending axons and dendrites to establish cell-cell interactions (Supplemental Fig. 1A) (Chen et al., 2013). To examine SULT4A1 expression, protein and RNA extracts were prepared from 1, 3, 5, and 7 days of culture after initial seeding for subsequent Western blot and real-time PCR analyses, respectively (Fig. 2). SULT4A1 protein was barely detected at day 1 and increased as culture continued, which correlated with neuron maturation (Fig. 2A). Similar to SULT4A1, SULT2B1 protein increased as neurons matured. SULT1A1 and SULT1E1 were not detected at any time points of culture (Fig. 2A). Real-time PCR analysis revealed that SULT4A1 mRNA levels did not correlate with its protein levels; mRNA was already expressed before its protein was detected and peaked at 5 days, whereas protein levels peaked at 7 days (Fig. 2B). To examine this discrepancy, SULT4A1 transcripts were analyzed on an agarose gel (Fig. 2C). One splice variant and wild-type mRNAs were detected. Variant was expressed during the initial period of culture. Reciprocal to variant decrease, wild type increased in a time-dependent manner. This increase of wild type appeared to correlate with protein levels. The variant mRNA contains a pseudo exon 6 and translates a

Fig. 2. SULT4A1 expression during neuron maturation. (A) Mesencephalic neuron-glia cells were cultured as described in the *Materials and Methods* section. Western blots were performed as described in Fig. 1. A representative image of three independent experiments was indicated. (B) Real-time PCR. Total RNAs were extracted from each stage of neuron glia cultures. (C) SULT4A1 mRNAs were amplified by reverse-transcription PCR. (D) Real-time PCR. SULT2B1 mRNAs were amplified as indicated in (B). Each value is shown as the mean $\pm$ S.D. ($n = 3$). Asterisks indicate significant differences between experimental groups (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). WT, wild type.

truncated/inactive enzyme (Sidharthan et al., 2014). These observations were consistent with previous findings with rat brains and human neuroblastoma cell lines (Falany et al., 2000; Sidharthan et al., 2014). SULT2B1 mRNA increased as its protein increased (Fig. 2D). No mRNAs were detected for SULT1A1 and SULT1E1 in neuron-glia mixed cells at any time points (data not shown). Although SULT4A1 was found not to be expressed in glia cells in our present work, it was previously reported in glia-derived cell lines (Sidharthan et al., 2014). To examine this difference, Western blot analysis was used to examine the expression in glioblastoma-derived U373-MG cells and dopaminergic neuron-derived N27 cells. SULT4A1 could not be detected in these immortalized cells (data not shown).

SULT4A1 Expression Upon Chemical Treatments. Neuron-enriched cultures were treated with various exogenous chemicals and endogenous hormones for subsequent Western blot analysis of SULT4A1. Since Toll-like receptor signaling is known to regulate axonal growth and neuronal plasticity (Okun et al., 2011), neuron cultures were treated with increased concentrations of lipopolysaccharide (Fig. 3A). Nuclear receptors such as constitutive androstane receptor, pregnane X receptor, and farnesoid X receptor are known to regulate expressions of SULTs (Kodama and Negishi, 2013). Neuron-enriched cultures were treated with their activators and ligands such as phenobarbital, CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime), rifampicin, and GW4064 (3-(2,6-Dichlorophenyl)-4-(3'-carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole). None of these tested affected levels

of SULT4A1 (Fig. 3B). Growth factors epidermal growth factor, hepatocyte growth factor, endocrine hormones 3,3',5-triiodo-L-thyronine, pregnenolone, DHEA, and DHEA-sulfate did not alter SULT4A1

Fig. 3. Effects of various compounds on SULT4A1 expression in mouse primary neurons. (A) Mouse neuron-enriched cells were treated with lipopolysaccharide (LPS; 1–15 $\mu\text{g/ml}$) for 24 hours and harvested for Western blot analysis. (B) The cells were treated with epidermal growth factor (EGF; 10 ng/ml), hepatocyte growth factor (HGF; 50 ng/ml), phenobarbital (PB; 1 mM), CITCO (1 μM), rifampicin (RIF; 10 μM), GW4064 (5 μM), forskolin (10 μM), 3,3',5-triiodo-L-thyronine (T3; 10 nM), pregnenolone (PREG; 500 nM), DHEA (100 nM), or DHEA-sulfate (DHEAS; 100 nM) for 24 hours, followed by Western blot analysis. A representative Western blot image of three independent experiments was indicated. DMSO, dimethylsulfoxide.

levels. Moreover, a protein kinase A activator, forskolin, also showed no effect (Fig. 3B).

Immunofluorescence Staining of Neurons and Glia for SULT4A1. Mixed neuron-glia cells were cultured for 7 days for subsequent double immunofluorescence staining by an anti-SULT4A1 antibody with an antineuronal marker NeuN antibody, an antidopaminergic neuron marker TH antibody, an astrocyte marker glial fibrillary acidic protein antibody, or a microglia marker IBA-1 antibody. All neurons stained by an anti-NeuN antibody were also stained by an anti-SULT4A1 antibody (Fig. 4A). About 2% of cultured cells are dopaminergic neurons (Gao et al., 2002). There were two dopaminergic neurons in this section, both of which were costained by an anti-SULT4A1 antibody (Fig. 4B). Neither astrocytes nor microglia were costained (Fig. 4, C and D), confirming that SULT4A1 was not expressed in glia cells.

Discussion

As for glia cells, astrocytes produce and provide energy sources to other brain cells, whereas microglia are resident macrophages and are the only immune cells in the brain. The finding that SULT4A1 was not expressed in either glia cell indicates that SULT4A1 is not involved in their respective cell functions. On the other hand, SULT4A1 may play roles in the neuronal maturation and/or in protecting neurons from

degeneration. Since SULT4A1 knockout mice survive through embryonic stages (Garcia et al., 2018), mixed neuron-glia cells can be prepared from E14 embryos from this mutant mouse, providing us with an excellent experimental system to determine physiologic roles of SULT4A1 in neurons.

Since ablation of the *Sult4a1* gene caused severe neuronal abnormalities (Garcia et al., 2018), SULT4A1 must critically regulate various neuronal functions. In this respect, our finding of SULT4A1 expression in dopaminergic neurons is informative, since abnormalities of dopaminergic neurons have been associated with the development of various neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's diseases (Block and Hong, 2005; Cepeda et al., 2014). Neuroinflammation mediated through dysregulated microglia has been linked to the pathogenesis of Parkinson's disease (Gao et al., 2002). However, since SULT4A1 is not expressed in microglia, if SULT4A1 has a direct role in these neuronal degenerations, it is not initiated from microglia. Whereas neuron-specific expression of SULT4A1 is an informative finding, SULT1E1 not being expressed in brains and neurons is a surprising but equally informative finding. The brain is a major estrogen-targeted organ (Gillies and McArthur, 2010). In addition to exciting numerous physiologic functions, estrogens are also toxic chemicals that adversely affect organs. SULT1E1 is the enzyme that inactivates estrogen. Sulfated estrogen can be reactivated through desulfation by steroid sulfatase (Purohit et al., 1994). Thus, SULT1E1 is ubiquitously expressed as a key enzyme that controls the metabolic balance

Fig. 4. Double fluorescence staining of mouse primary neuron/glia cells. Mesencephalic primary neuron-glia cells were prepared from E14 mouse embryos and cultured for 7 days. Cells were double stained by an anti-SULT4A1 antibody with NeuN (A), TH (B), glial fibrillary acidic protein (GFAP) (C), or IBA-1 (D). Nuclei was stained by 4',6-diamidino-2-phenylindole (DAPI).

of estrogen activity, which is intriguing because is not expressed in the brain. Although SULT1A1 and SULT2A1 were reported to sulfate estrogen, but were poorly compared with SULT1E1 (Harris et al., 2000; Wang and James, 2005), they were not expressed in mouse primary neurons (Fig. 1A) and brains (Salman et al., 2011), respectively. As for estrogen sulfation, it was reported that SULT4A1 bound 2-hydroxyestradiol in thermostability screening assays (Allali-Hassani et al., 2007). Moreover, SULT4A1 and estrogen receptor α concomitantly increased their expression levels in neurons during maturation (Supplemental Fig. 1B), and our preliminary work with recombinant SULT4A1 enzyme suggested that it may sulfate estrogen (unpublished data). However, SULT4A1, being an enzyme that sulfates estrogen, is uncertain now and remains to be explored in further investigations.

Expression of SULT4A1 increased in primary neurons as they matured and was higher in adult than neonate brains. Two forms of SULT4A1 mRNA, wild type and a splice variant, were expressed in primary neurons, of which the increase of the wild type correlated with that of the SULT4A1 protein. However, the cell signal mechanism that developmentally activates the *Sult4a1* gene remains uninvestigated. Nearly 10 endobiotics and exogenous chemicals were examined, none of which affected SULT4A1 expression in neurons. Finding the regulatory mechanism of this expression should help us to understand the neuronal function of SULT4A1.

In conclusion, SULT4A1 appears to play a role in maturation of neurons. Expression of SULT4A1 in dopaminergic neurons is indicative of its involvement in neuronal functions, such as movements. Although SULT4A1 may regulate both beneficial and adverse effects of estrogens, the involvement of SULT4A1 in estrogen metabolism remains an urgent subject of future investigations.

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