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In vitro evidence that sulfite impairs glutamatergic neurotransmission and inhibits glutathione metabolism-related enzymes in rat cerebral cortex

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ABSTRACT

Sulfite oxidase (SOX) deficiency is an inherited neurometabolic disorder biochemically characterized by tissue accumulation and high urinary excretion of sulfite and thiosulfate. Affected patients present severe neurological dysfunction accompanied by seizures, whose pathophysiology is poorly known. In the present study we evaluated the *in vitro* effects of sulfite and thiosulfate on important parameters of glutamatergic neurotransmission and redox homeostasis in rat cerebral cortex slices. We verified that sulfite, but not thiosulfate, significantly decreased glutamate uptake when cerebral cortex slices were exposed during 1 h to these metabolites. We also observed that thiosulfate inhibited glutamine synthetase (GS) activity. A pronounced trend toward GS inhibition induced by sulfite was also found. Regarding redox homeostasis, sulfite, at the concentration of 10 μM, increased thiobarbituric acid-reactive substances and decreased glutathione concentrations after 1 h of exposure. In contrast, thiosulfate did not alter these parameters. We also found that 500 μM sulfite increased sulfhydryl group content in rat cerebral cortex slices and increased GSH levels in a medium containing oxidized GSH (GSSG) and devoid of cortical slices, suggesting that sulfite reacts with disulfide bonds to generate sulfhydryl groups. Moreover, sulfite and thiosulfate did not alter the activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione *S*-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH) after 1 h of incubation. However, sulfite inhibited the activities of GPx, GST and G6PDH when cortical slices were exposed for 3 h to sulfite. We finally verified that sulfite did not induce cell death after 1 h of incubation. Our data show that sulfite impairs glutamatergic neurotransmission and redox homeostasis in cerebral cortex. Therefore, it may be presumed that these pathomechanisms contribute, at least in part, to the seizures observed in patients affected by SOX deficiency.

1. Introduction

Sulfite oxidase (SOX) deficiency is a debilitating disease caused either by a mutation in the gene encoding the enzyme protein (isolated SOX deficiency) or by defects in the pathway that synthesize its molybdopterin cofactor (molybdenum cofactor deficiency). SOX catalyses the oxidation of sulfite to sulfate, the final step in the pathway of degradation of the sulfur-containing amino acids cysteine and methionine (Johnson and Duran, 2001; Schwarz et al., 2009). Sulfite may also be derived from exogenous sources, such as sulfiting agents that are used as food and medication preservatives (Taylor et al., 1986). Both forms of SOX deficiency (isolated SOX

Abbreviations: SOX, sulfite oxidase; GS, glutathione sinthetase; TBA-RS, thiobarbituric acid-reactive substances; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-*S*-transferase; G6PDH, glucose-6-phosphate dehydrogenase; DNPH, 2,4-dinitrophenylhydrazine; CDNB, 1-chloro-2,4-dinitrobenzene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DTNB, 5,5-dithio-bis (2-nitrobenzoic acid); GSSG, oxidized glutathione; LDH, lactate dehydrogenase; GLAST, glutamate/aspartate transporter; GLT1, glutamate transporter 1; EAAC1, excitatory amino acid carrier 1.

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deficiency and molybdenum cofactor deficiency) are biochemically characterized by an abnormal tissue accumulation and high urinary excretion of sulfite, thiosulfate and cysteine-*S*-sulfate. Patients with the cofactor deficiency additionally present increased levels of xanthine and hypoxanthine, since the enzyme xanthine dehydrogenase is also defective in this condition (Johnson and Duran, 2001; Schwarz et al., 2009).

The clinical presentation of SOX deficiency consists of severe neurological symptoms, including seizures and mental retardation that may lead to premature death (Bindu et al., 2011). Neuropathological analysis reveals severe encephalopathy with neuronal death and demyelination accompanied by gliosis and diffuse spongiosis in the cerebral cortex. Marked atrophy of the basal ganglia and thalami, and dilated brain ventricles are also reported in MRI (Basheer et al., 2007; Dublin et al., 2002; Johnson and Duran, 2001).

Previous reports showed that the accumulating metabolites in SOX deficiency are neurotoxic. In this context, a modulation of NMDA receptor subunits was demonstrated in hippocampus of SOX-deficient rats fed with sulfite (Oztürk et al., 2006). Other studies suggested that cysteine-*S*-sulfate could induce excitotoxicity due to its chemical similarity to glutamate (Salman et al., 2002; Tan et al., 2005). Previous data verified that sulfite and thiosulfate markedly inhibit the activity of the enzyme creatine kinase, whereas sulfite also causes brain mitochondrial dysfunction (Grings et al., 2013; Zhang et al., 2004). In addition, sulfite generates reactive oxygen species (ROS) via autooxidation reactions leading to lipid peroxidation and impairment of antioxidant defenses in rat brain (Chiarani et al., 2008; Kocamaz et al., 2012; Küçükatay et al., 2005; Niknahad and O'Brien, 2008; Ozsoy et al., 2014).

Considering that the mechanisms involved in the brain injury observed in SOX deficiency are still unclear and that practically nothing has been studied concerning glutamatergic neurotransmission, the present study evaluated the *in vitro* effects of sulfite and thiosulfate on glutamate uptake, glutamine synthetase (GS) activity, thiobarbituric acid-reactive substances (TBA-RS) levels, glutathione (GSH) concentrations, sulfhydryl content, carbonyl formation and the activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione *S*-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH). The effect of sulfite on cell viability and integrity were also determined. These parameters were analyzed in rat cerebral cortex slices, which consist in a system with preserved cell machinery, including enzymes, as well as glutamate transporters and receptors.

2. Experimental procedures

2.1. Chemicals

Reagents were mainly purchased from Sigma Chemical Co., St. Louis, MO, USA, except for [^{3}H]glutamate (49.6 Ci/mmol), which was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA).

2.2. Animals

Thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil, were used. The animals had free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil) and were maintained on a 12:12 h light/dark cycle in an air conditioned constant temperature (22 °C ± 1 °C) colony room. The experimental protocol was approved by the Ethics Committee for Animal Research of UFRGS, Porto Alegre, Brazil, and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

2.3. Sample preparation for glutamatergic system and MTT reduction evaluation

Animals were euthanized by decapitation, the brain was immediately removed and sliced using a McIlwain tissue chopper. The slices (400 μm) were submerged in Na^+-free medium for the measurement of glutamate uptake; in Krebs-bicarbonate buffer, pH 7.4, for GS activity determination; or in HBSS for MTT reduction assay. Then, cerebral cortex slices were isolated, transferred to a 48-well plate (one slice per well) and incubated with sulfite or thiosulfate (10–500 μM) at 37 °C during 1 h. After incubation, the slices were used for glutamate uptake determination and MTT reduction or homogenized in 200 μL of Krebs-bicarbonate buffer to measure GS activity.

2.4. Sample preparation for oxidative stress parameters evaluation

Rats were euthanized, had their brain quickly removed and placed on an ice plate. Cerebral cortex was dissected and sliced with a McIlwain tissue chopper. Approximately 60–75 mg of cortical slices (300 μm) were placed in each well of a 48-well plate and incubated with sulfite or thiosulfate (10–500 μM) at 37 °C for 1 h. After incubation, the slices were homogenized in 10 volumes (1:10, w/v) of Krebs-bicarbonate buffer, pH 7.4. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant separated to evaluate the biochemical parameters.

2.5. Glutamate uptake

Glutamate uptake experiments were carried out in the presence of high Na^+ concentrations, which favor glutamate binding to high-affinity uptake carriers (Almeida et al., 2010; Frizzo et al., 2002). We used Hank's buffered salt solution (HBSS) containing 137 mM NaCl, 0.63 mM Na_2HPO_4, 4.17 mM $NaHCO_3$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.40 mM $MgSO_4$, 0.50 mM $MgCl_2$, and 5.5 mM glucose, pH 7.2, for measuring this uptake. After the incubation of cortical slices in the presence of the metabolites, we added 0.33 μCi/mL [^{3}H]glutamate and 100 μM nonradioactive glutamate. The reaction was stopped after 7 min with five ice-cold washes with 1 mL HBSS and solubilized overnight with 200 μL of 0.5 M NaOH. Na^+-independent uptake was determined by using *N*-methyl-D-glucamine instead of sodium chloride. Na^+-dependent uptake was calculated as the difference between glutamate uptakes measured in the medium containing Na^+ and in the medium containing *N*-methyl-D-glucamine (Frizzo et al., 2002). The radioactivity incorporated was determined in a Wallac 1409 liquid scintillation counter. Results are expressed in nmol/mg of protein.

2.6. Glutamine synthetase (GS) activity

GS activity was determined in homogenates obtained from cortical slices after incubation as previously described (Olney, 1969). One hundred microliters of homogenate were added to a reaction mixture containing 50 mM imidazole buffer, pH 6.8, 100 mM glutamine, 50 mM hydroxylamine, 25 mM sodium arsenate, 2 mM $MgCl_2$ and 0.2 mM ADP and incubated for 15 min at 37 °C. The reaction was stopped by the addition of a solution containing 370 mM ferric chloride, 100 mM HCl and 50 mM trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 540 nm and compared to a calibration curve of