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Volatile anesthetics attenuate oxidative stress-reduced activity of glutamate transporter type 3

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Abstract

Background—Volatile anesthetics enhance the activity of glutamate transporter type 3 (also called excitatory amino acid transporter type 3, EAAT3), the major neuronal EAAT. In addition to glutamate, EAAT3 can also uptake L-cysteine, the rate-limiting substrate for the synthesis of glutathione. Our previous study showed that oxidative stress inhibited glutamate-induced EAAT3 activity. We determined whether oxidative stress would reduce L-cysteine-induced EAAT3 activity and whether this reduction would be attenuated by volatile anesthetics.

Methods—Rat EAAT3 was expressed in *Xenopus* oocytes. L-glutamate- and L-cysteine-induced membrane currents were recorded using the two-electrode voltage clamp technique. The peak current was quantified to reflect the amount of transported substrates because transport of substrates via EAATs is electrogenic.

Results—Exposure of oocytes to 5 mM *tert*-butyl hydroperoxide, an organic oxidant, for 10 min reduced the Vmax, but did not affect the Km, of EAAT3 for L-cysteine. The volatile anesthetics isoflurane, sevoflurane and desflurane at concentrations from 1 to 3% attenuated the *tert*-butyl hydroperoxide-reduced EAAT3 activity for L-glutamate and L-cysteine.

Conclusions—Our results suggest that volatile anesthetics preserve EAAT3 function to transport L-glutamate and L-cysteine under oxidative stress, which may be a mechanism for the neuroprotective effects of volatile anesthetics.

Introduction

Oxidative stress and injury contribute significantly to pathophysiology of many human diseases, such as neurodegenerative diseases and ischemia-reperfusion injury.[1–3] Reducing oxidative injury has been recognized as an effective approach to protect cells during these disease processes.

Glutamate transporters, also named excitatory amino acid transporters (EAAT), uptake glutamate, the major excitatory neurotransmitter in the central nervous system and, therefore, can regulate glutamate neurotransmission and prevent glutamate neurotoxicity.[4] EAATs also transport cysteine, especially the EAAT type 3 (EAAT3) that is the major glutamate transporter

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in neurons.[5] Cysteine is the rate-limiting substrate for the synthesis of glutathione, the major intracellular antioxidant. It has been shown that EAAT3 deficient cells have decreased levels of glutathione and increased vulnerability to oxidants.[6] Thus, EAAT3 may play an important role in reducing oxidative stress and injury.

Volatile anesthetics including isoflurane provide protection against ischemia-reperfusion injury in various organs such as brain.[7] Isoflurane has also been shown to reduce oxidative stress-induced cell injury.[8] However, isoflurane does not have direct anti-oxidant effects.[9] Thus, volatile anesthetics may work through indirect mechanisms to reduce oxidative stress-induced cell injury. We have shown that isoflurane increases EAAT3 activity[10,11] and that oxidative stress can reduce glutamate transport via EAAT3.[12] Thus, we hypothesize that oxidative stress reduces cysteine transport via EAAT3 and that volatile anesthetics attenuate this reduction. To test these hypotheses, we expressed EAAT3 in *Xenopus* oocytes and examined L-glutamate- and L-cysteine-induced membrane currents.

Materials and Methods

Oocyte Preparation and Expression of Excitatory Amino Acid Transporter Type 3

The study protocol was approved by the Institutional Animal Care and Use Committee at the University of Virginia (Charlottesville, VA). Mature female *Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbor, MI) and fed with regular frog brittle twice weekly. For removal of oocytes, frogs were anesthetized with 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO) in water until unresponsive to painful stimuli (toe pinching) and underwent surgery on ice. A 5-mm incision was made in the lower lateral abdominal quadrant, and a lobule of ovarian tissue, containing approximately 200 oocytes, was removed and placed immediately in modified Barth solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.1 mM gentamicin and 15 mM HEPES, pH adjusted to 7.6. The oocytes were defolliculated with gentle shaking for approximately 2 h in calcium-free oocyte Ringer 2 solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES and 0.1% collagenase type Ia, pH adjusted to 7.5 and then kept in modified Barth's solution at 16°C.

The rat EAAT3 complementary DNA (cDNA) construct was provided by Dr. M.A. Hediger (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA). The cDNA was subcloned into a commercial vector (BluescriptSKm). The plasmid DNA was linearized with a restriction enzyme (Not I). mRNA was synthesized *in vitro* using a commercially available kit (Ambion, Austin, TX). The resulting mRNA was quantified spectrophotometrically and diluted in sterile water. This mRNA was used for the cytoplasmic injection of oocytes in a concentration of 40 ng/40 nl using an automated microinjector (Nanoject; Drummond Scientific Co., Broomall, PA). Each oocyte received an injection of 40 nl mRNA solution. Oocytes were then incubated at 16°C in modified Barth's solution for 3 days before voltage-clamping experiments.

Electrophysiological Recording

Experiments were performed at room temperature (approximately 21–23°C). A single oocyte was placed in a recording chamber (0.5 ml volume) and perfused with 4 ml/min Tyrode solution containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM dextrose, and 10 mM HEPES, pH adjusted to 7.5. Clamping microelectrodes were pulled from capillary glass (10 μl Drummond microdispenser, Drummond Scientific) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). Tips were broken to a diameter of approximately 10 μm. These microelectrodes provided resistance of 1–3 MΩ when they were filled with 3 M KCl. The oocytes were voltage-clamped using a two-microelectrode oocyte

voltage clamp amplifier (OC725-A; Warner Corporation, New Haven, CT) connected to a data acquisition and analysis system running on a personal computer. The acquisition system consisted of a DAS-8A/D conversion board (Keithley-Metrabyte, Taunton, MA). Analyses were performed with pCLAMP7 software (Axon Instruments, Foster City, CA). All measurements were performed at a holding potential of −70 mV. Oocytes that did not show a stable holding current less than 1 μA were excluded from analysis. L-glutamate and L-cysteine were diluted in Tyrode solution and superfused at 5 ml/min over the oocyte for 20 s. Experimental chemical–induced currents were sampled at 125 Hz for 1 min: 5 s of baseline, 20 s of L-glutamate or L-cysteine application and 35 s of washing with Tyrode solution. The L-glutamate- or L-cysteine–induced peak currents were calculated to reflect the amount of transported L-glutamate- or L-cysteine because transport of substrates via EAATs is electrogenic. The sizes of currents induced by their substrates can be used to reflect the amount of substrates transported via EAATs except for EAAT4 that can have a large substrate-induced chloride current that is not thermodynamically coupled to substrate transport.[4,13,14]

Administration of Experimental Chemicals and Anesthetics

A reservoir filled with 40 ml Tyrode solution was bubbled by output from a calibrated anesthetic-specific vaporizer. The carrier gas was air flowing at 500 ml/min. Ten min were allowed for equilibration. In the control group, oocytes were perfused with Tyrode solution for 4 min before the responses were measured. In the volatile anesthetic group, oocytes were perfused with Tyrode solution for the first 1 min, followed by Tyrode solution equilibrated with volatile anesthetics for the next 3 min before the response measurement. We tested isoflurane, sevoflurane, and desflurane, three commonly used volatile anesthetics. To study L-cysteine-induced dose-response of EAAT3 activity, oocytes were exposed to serial concentrations of L-cysteine (3, 10, 30, 100, 300, 1000 and 3000 μM). Oxidative stress was induced by preincubating oocytes for 10 min in modified Barth's solution containing 5 mM of *tert*-butyl hydroperoxide (*t*-BHP). This concentration was used because the IC_{50} for *t*-BHP to inhibit glutamate-induced responses in EAAT3 was 4.1 mM in our previous study.[12]

Chemicals

Isoflurane and desflurane were purchased from Baxter Healthcare Corporation (Deerfield, IL) and sevoflurane was purchased from Abbott Laboratories (North Chicago, IL). Other chemicals were obtained from Sigma (St. Louis, MO).

Data Analysis

Results under various experimental conditions were normalized to the mean value of the same day controls obtained from the same batch of oocytes due to the concern of various EAAT protein expression levels on different days and from different batches of oocytes. Responses in the L-cysteine dose-response experiments were normalized to the mean responses in the presence of 3 mM L-cysteine also on the same day and from the same batch of oocytes. Each experimental condition was repeated with oocytes from at least 4 frogs. Results are reported as means ± S.D. Statistical analysis of the results in the L-cysteine dose-response experiments was performed by t-test and the other results were analyzed by one-way analysis of variance followed by the Tukey test. A $P < 0.05$ was considered significant.

Results

As we showed before,[13] L-glutamate induced an inward current in oocytes injected with EAAT3 mRNA and did not induce any current in oocytes without injection of EAAT3 mRNA, suggesting that the inward current induced by L-glutamate is mediated by EAAT3. Similarly, L-cysteine induced an inward current only in oocytes injected with EAAT3 mRNA.

Previous studies have shown that the Km of EAAT3 for L-glutamate is about 30 μM.[13,15] Thus, we used 30 μM L-glutamate in this study. Similar to our previous findings,[12] *t* -BHP, an organic oxidant, decreased L-glutamate-induced responses. This decreased response was attenuated by various concentrations of isoflurane, sevoflurane, and desflurane. Interestingly, a clear dose-dependent response of this volatile anesthetic effect was not apparent with the range of concentrations studied (1 – 3%) (Fig. 1). Isoflurane, sevoflurane, and desflurane at 1% significantly attenuated the *t*-BHP-reduced response of EAAT3 to L-glutamate, suggesting that low concentrations of these anesthetics can attenuate the t-BPH reduction of EAAT3 activity.

We showed that *t*-BHP reduced the Vmax, but did not change the Km, of EAAT3 for L-glutamate.[12] In this study, 5 mM *t*-BHP reduced the Vmax (100 ± 19% under control condition and 65 ± 10% with *t*-BHP, $P < 0.05$), but did not affect the Km (89 ± 22 μM under control condition and 83 ± 22 μM with *t*-BHP, $P > 0.05$), of EAAT3 for L-cysteine (Fig. 2). Thus, we used 100 μM L-cysteine in the subsequent studies.

The responses of EAAT3 to L-cysteine were significantly decreased by 5 mM *t*-BHP. Similar to the results with L-glutamate, the decreased transport of L-cysteine was partially attenuated by various concentrations of isoflurane, sevoflurane, or desflurane (Fig. 3), an effect that was statistically significant for all three volatile anesthetics.

Discussion

Many biological processes produce reactive oxygen species (ROS), which are normally neutralized by antioxidants such as glutathione. ROS can oxidize proteins, DNA, and lipids, altering the normal functions of these substances. Oxidative stress-induced injury is a major component of ischemia-reperfusion injury.[1] Oxidative injury also contributes to pathophysiology of many common neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.[3,16] Oxidative stress may be a major mechanism for aging-induced brain functional and structural changes.[17] Thus, oxidative stress and injury are fundamental mechanisms underlying a broad range of human diseases. Reducing oxidative injury may be an effective approach to protecting cells and mitigating these disease processes.

In this study oxidative stress reduced L-cysteine-induced EAAT3 activity. Thus, a vicious cycle may occur during oxidative stress: oxidative stress consumes glutathione, which is not replaced because the same oxidative stress reduces cysteine transport via EAAT3, further exacerbating the oxidative stress. We also showed that *t*-BHP-reduced EAAT3 responses to L-cysteine were mainly due to the decreased Vmax. The Km of EAAT3 for L-cysteine was not affected by *t*-BHP. These results suggest that *t*-BHP reduces the amount of EAAT3 available for L-cysteine transport in the cell surface but does not affect the affinity of EAAT3 for L-cysteine.

We have previously shown that volatile anesthetics increase EAAT3 transport of glutamate.[13] The mechanisms for isoflurane-induced increase in EAAT3 activity include an increased amount of EAAT3 proteins in the plasma membrane,[10] the functional site for EAAT3 to transport its substrates. This mechanism is in opposite direction to the *t*-BHP effects on EAAT3. Thus, we hypothesized that inhaled anesthetics should attenuate *t*-BHP effects on EAAT3, as shown here by the ability of isoflurane, sevoflurane, and desflurane to increase EAAT3 responses to L-glutamate and L-cysteine in the presence of *t*-BHP.

The role of EAAT3 in anti-oxidative stress in the brain has been well studied. EAAT3 binds and transports cysteine far more effectively than EAAT2 and EAAT1.[18] This feature, along with the fact that EAAT3 is distributed in the cell body of neurons,[19] suggests that cysteine uptake by EAAT3 may be a major mechanism for supplying the substrate for the synthesis of glutathione in neurons. This suggestion is supported by the lack of cystine-glutamate exchanger

(X_c^-) expression in neurons.20 Hetero-exchange of cystine with glutamate via X_c^- is a way to acquire cysteine in the form of cystine in most cell types. A recent study showed that EAAT3 knockout mice had reduced neuronal glutathione levels and, with aging, developed brain atrophy and decreased learning and memory functions.[6] The hippocampi of these mice also had increased oxidant levels and enhanced susceptibility to oxidant injury.[6] These data suggest a critical role of EAAT3 in maintaining neuronal glutathione levels to reduce oxidant-induced neuronal injury and death.

Volatile anesthetics have been reported to have neuroprotective effects.[7,21] The preservation of EAAT3 function under oxidative stress may contribute to their neuroprotection by disrupting the vicious cycle leading to decreased intracellular glutathione level under oxidative stress. Since oxidative stress has been implicated in various human diseases,[1,3,16] preserving the EAAT3 function under oxidative stress by various agents including volatile anesthetics may have broad implications.

Our study has limitations. We used an oocyte expression system so that we could express single type of EAAT. Although oocytes have components of all major intracellular signaling pathways of mammalian cells and have been used for studies of EAAT activity,[22,23] oocyte expression system provides an artificial environment for EAATs. Thus, we must be cautious in extrapolating the results from oocytes to mammalian neurons.

In summary, oxidative stress decreases EAAT3 responses to L-glutamate and L-cysteine by decreasing Vmax. Volatile anesthetics attenuated the effect of oxidative stress on EAAT3 responses to L-glutamate and L-cysteine.

Acknowledgments

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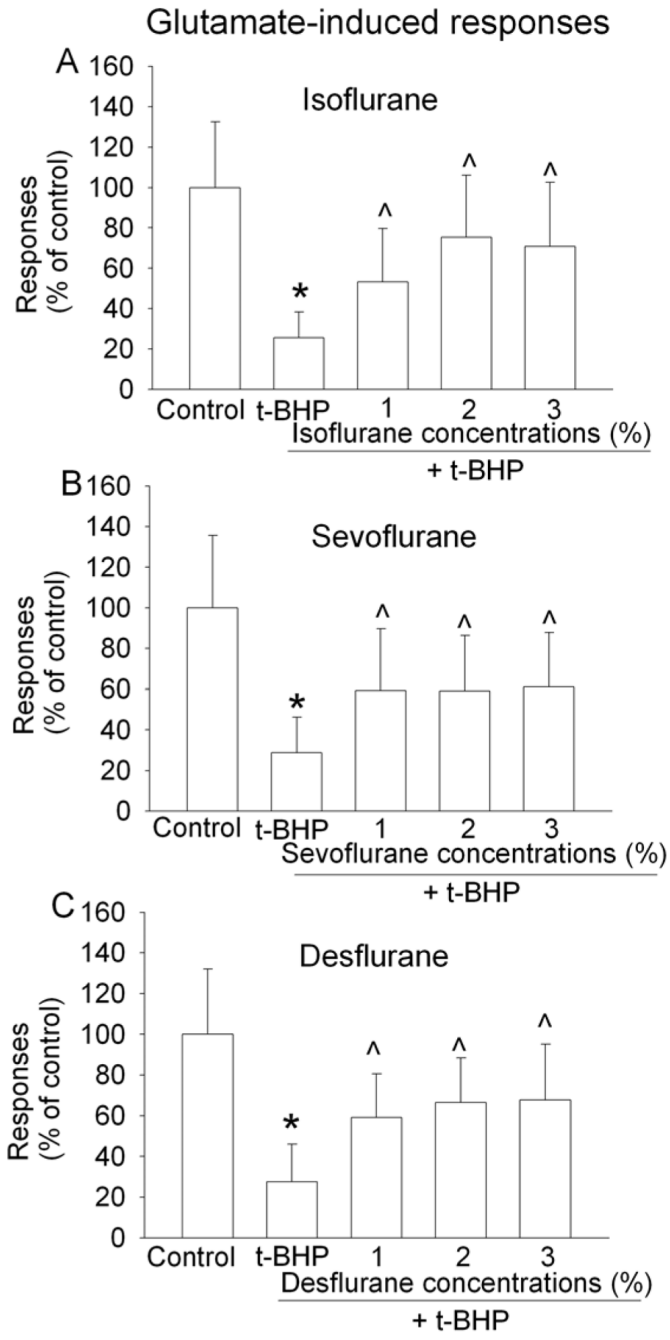

Fig. 1. Effects of volatile anesthetics on tert-butyl hydroperoxide (t-BHP)-reduced responses of excitatory amino acid transporter type 3 (EAAT3) to L-glutamate

Oocytes were exposed to 5 mM *t*-BHP for 10 min at room temperature and then the responses to 30 μM L-glutamate were recorded. Results are means ± S.D. (n = 17 – 29 for isoflurane, 18 – 32 for sevoflurane, and 13 – 28 for desflurane). * $P < 0.05$ compared with the control. ^ $P < 0.05$ compared with *t*-BHP only.

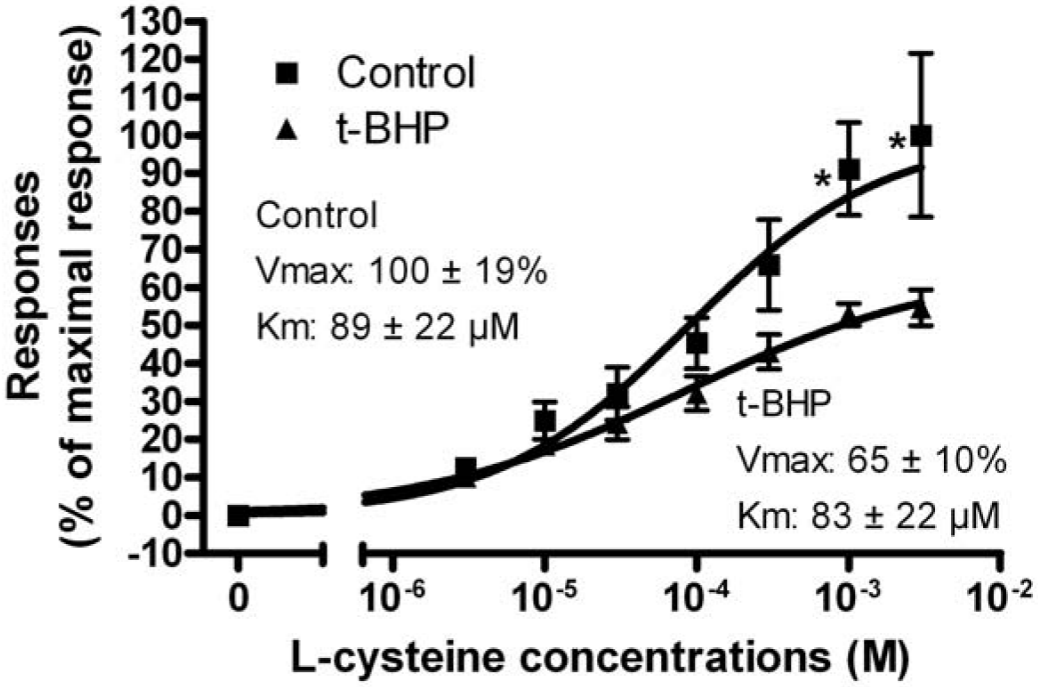

Fig. 2. Concentration-responses of excitatory amino acid transporter type 3 (EAAT3) to L-cysteine in the presence or absence of 5 mM tert-butyl hydroperoxide (t-BHP)

Oocytes were exposed to5 mM *t*-BHP for 10 min at room temperature and then the responses to various concentrations of L-cysteine were recorded. Results are means ± S.D. (n = 9). * $P < 0.05$ compared with the corresponding values in the presence of 5 mM *t*-BHP.

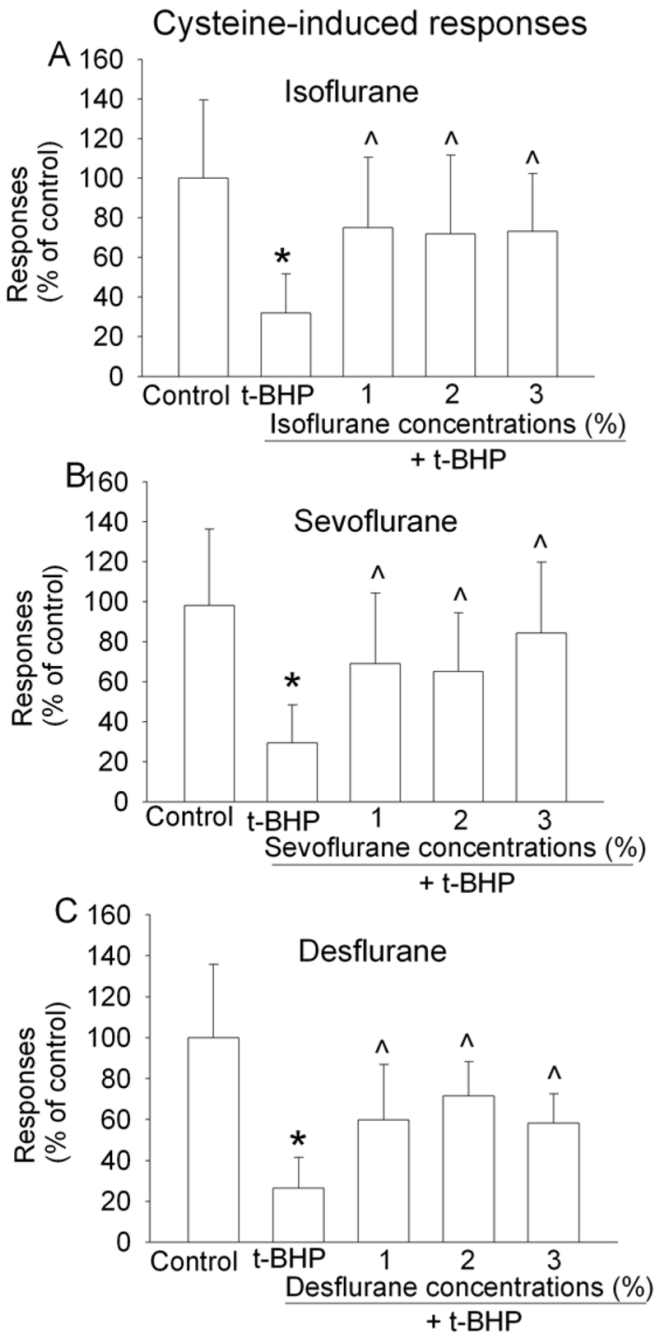

Fig. 3. Effects of volatile anesthetics on tert-butyl hydroperoxide (t-BHP)-reduced responses of excitatory amino acid transporter type 3 (EAAT3) to L-cysteine

Oocytes were exposed to 5 mM *t*-BHP for 10 min at room temperature and then the responses to 100 μM L-cysteine were recorded. Results are means ± S.D. (n = 16 – 29 for isoflurane, 13 – 29 for sevoflurane, and 13 – 27 for desflurane). * $P < 0.05$ compared with the control. ^ $P < 0.05$ compared with *t*-BHP only.