Anesthesiology 2009; 110:538–47

Role for Metallothioneins-I/II in Isoflurane Preconditioning of Primary Murine Neuronal Cultures

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Background: **Pretreatment with inhaled anesthetics, including isoflurane, can induce long-lasting cellular protection against ischemia-derived toxicity in multiple tissues, including brain tissue. Metal-regulatory genes, metallothioneins-I/II (MT-I/II), have been shown to protect against oxidative damage in multiple tissues. Furthermore, MT have been found to be differentially regulated in response to isoflurane and ischemic preconditioning. In this study, we assess the role of MT-I/II in mediating isoflurane preconditioning in primary neuronal-glial cultures.**

Methods: **Primary mouse neuronal-glial cultures were preconditioned with isoflurane (3 h, 1.5%) 24–96 h before 3-h oxygen-glucose deprivation (OGD, ischemic model). After OGD, isoflurane protection and responsiveness of MT-I/II knockdown and knockout cultures to preconditioning were assessed by lactate dehydrogenase release. Immunoassays for microtubule associated protein 2 and glial fibrillary acidic protein determined neuronal-glial sensitivity to preconditioning. MT-I/II messenger RNA was assessed by quantitative reverse transcriptase-polymerase chain reaction. Cultures transfected with exogenous MT-I/II were analyzed for protection against OGD toxicity.**

Results: **Isoflurane preconditioning reduced OGD-mediated toxicity by 11.6 ± 7.9% at 24 h, with protection increasing to 37.5 ± 2.5% at 72 h after preconditioning. Immunolabeling showed that neurons were more sensitive to OGD and more responsive to isoflurane preconditioning compared to glia. Quantitative reverse transcriptase-polymerase chain reaction showed MT-I/II messenger RNA were upregulated (approximately 2.5-fold) by isoflurane treatments. Also MT-I/II protein transfection significantly decreased OGD-mediated toxicity. Finally, knockdown and knockout of MT-I/II diminished and abolished isoflurane-mediated protection, respectively.**

Conclusions: **MT-I/II play an important role in isoflurane-mediated delayed preconditioning against OGD toxicity of neuronal and glial cells *in vitro*.**

PRECONDITIONING is a phenomenon whereby a mild physiological or pharmacological insult is able to trigger protection against a subsequent, more substantial insult. Brief ischemia[1] and exposure to inhaled anesthetics[2,3] are among the stimuli able to induce preconditioning in a number of tissues, including heart and brain. Both anesthetic preconditioning (APC) and ischemic preconditioning display biphasic protection with an early window (≤3 h) mediated primarily by posttranslational protein modifications.[4] A subsequent delayed window of protection (delayed preconditioning) develops hours to days after preconditioning in ischemic preconditioning[5] and has been shown to last 72–96 h *in vivo*.[6] Rats preconditioned with isoflurane subjected to left common carotid arterial ligation showed better histologic and neurologic outcomes at 1 month compared to non-preconditioned controls,[7] demonstrating long-term benefits of anesthetic preconditioning. As a result, ischemic preconditioning and anesthetic preconditioning have emerged as potentially powerful tools to reduce ischemia-induced cell death both during surgery and during the perioperative period.[8,9]

Given the potential for improved clinical outcomes, mechanisms of delayed preconditioning have been intensely studied.[10] Since first demonstrated in heart,[11] preconditioning after exposure to volatile anesthetics has now been described in a variety of tissues for both *in vivo*[2,12,13] and *in vitro*[2,14] rodent neuronal models. In these studies, inducible nitric oxide synthase,[2,13] intracellular calcium,[14]mitogen-activated protein kinase,[13,14] and a variety of mitochondria-related proteins[15] have all been implicated in the cellular mechanisms of APC. Despite these findings, a complete understanding of the pathways involved in APC has yet to be elucidated.

Recent work in our lab identified metallothionein-I/II genes (MT-I/II) as among those significantly regulated in rat liver, kidney, and heart after a 90-min 2% isoflurane exposure.[16] Furthermore, Carmel *et al.*[17] found MT-I/II messenger RNAs (mRNAs) were strongly and rapidly upregulated in response to ischemic preconditioning of rat spinal cord. MT-I/II are small (6000–7000 Da), cysteine-rich, metal-binding proteins, and they have been shown to protect against a wide range of stresses, including cardiac ischemia-reperfusion[18]and focal cerebral ischemia.[19] To date, the relationship between MT-I/II proteins and anesthetic preconditioning remains to be explored. In the current study, we examine protective characteristics of delayed isoflurane preconditioning in dissociated neuronal culture and the role that MT-I/II play in conferring APC-mediated protection.

Materials and Methods

Animal Care

C57BL/6J (C57), 129S7/SvImJ (129S, control strain for MT-I/II knockout mice), and 129S7/SvEvBrd-*Mt1*^{tm1Bri} *Mt2*^{tm1Bri} /J (MTKO, MT-I/II knockout strain) were purchased from Jackson Laboratories (Bar Harbor, ME), and

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Received from the Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts; and Department of Biological Sciences, Smith College, Northampton, Massachusetts. Submitted for publication March 30, 2008. Accepted for publication October 22, 2008. Supported by Blakeslee Fund, Smith College, Northampton, Massachusetts, and Wilens Fund, Smith College, Northampton, Massachusetts (to Mr. Edmands).

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breeding colonies were established. Animals were kept under a standard 12/12 light/dark cycle at room temperature and given access to food and water *ad libitum*. Animals were housed, and all experiments were carried out with protocols approved by Smith College Institutional Animal Care and Use Committee (Northampton, Massachusetts) according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Primary Neuronal Cultures

Neonatal mouse pups at postnatal day 1–3 (P1-3) were sacrificed by decapitation. Cortices were dissected out, minced, and placed in modified DMEM (80% Dulbecco's modified eagle medium; 10% fetal bovine serum, 10% F12 medium, and 10 μg/ml Pen/Strep). Tissue was digested in Trypsin/Hanks Balanced Salt Solution (1:1) for 25 min, quenched with 50% horse serum, and then triturated with a fire-polished Pasteur pipette. The cell suspension was centrifuged for 3 min, filtered through a Steriflip100 μM nylon filter (Millipore, Billerica, MA), and plated onto polylysine-coated wells; in the case of immunofluorescence microscopy, polylysine-coated glass coverslips were used for plating. Cultures were maintained in an incubator (5% CO_2, 95% air) at 37°C for 10–12 days *in vitro*.

Isoflurane Preconditioning

Isoflurane preconditioning was accomplished by placing cultures (10–12 days *in vitro*) inside a humidified exposure chamber with inlet and exhaust ports for the anesthetic mix. The entire exposure chamber was housed within a 37°C incubator. A mixture of 95% air/5% carbon dioxide was passed through an Isotec 3 isoflurane vaporizer (GE Healthcare, Waukesha, WA) to deliver 1.5% isoflurane/93.5% air/5% CO_2 to the chamber at a rate of 0.4 l/min for 3 h. After APC, cells were washed, and 1× fresh medium was added before replacing cells in a standard humidified carbon dioxide incubator. For controls, all media changes were performed in parallel with preconditioned cultures.

Oxygen-Glucose Deprivation

Oxygen glucose deprivation (OGD) exposures were carried out as previously described[2] with some modifications. Cell cultures were washed 4 times with zero-glucose DMEM (Invitrogen, Carlsbad, CA) and placed in a humidified anoxic chamber at 37°C. The chamber was evacuated and flushed 3 times with 90% N_2, 5% H_2, and 5% CO_2 to remove oxygen. To further reduce oxygen levels, a palladium catalyst was also maintained in the chamber to react any remaining oxygen with the hydrogen. Non-OGD samples were treated the same as OGD-receiving samples, but they were washed with standard glucose-containing culture media.

QRT-PCR of MT-I/II RNA Levels

Cells were harvested using a cell scraper, pooled, pelleted by centrifugation, and then added to Trizol reagent (Invitrogen) and stored at −20°C for further processing. Total RNA for quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) analysis was isolated from Trizol/tissue homogenate using Qiagen Rneasy Mini kits (Qiagen, Valencia, CA). In brief, chloroform was added to the Trizol/tissue homogenate, and the aqueous phase was removed, mixed with 70% ethanol, and placed on a separation column. After repetitive washes and elution in RNA–free water, RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). All samples yielded 260/280 absorbance ratios between 1.9 and 2.1.

QRT-PCR of MT-I and MT-II mRNA was performed using a mouse-modified primer/probe set (FAM/TAMRA) described by Tomita *et al.*[20] Primers and probes were synthesized by Integreted DNA Technologies, Inc. (Coralville, IA): MT-I forward *5′-CTGCTCCACCGGCGG-3′*, MT-1 Reverse *5′-GCCCTGGGCACATTTGG-3′*; MT-I taqman probe*: 5′-CTCCTGCAAGAAGAGCTGCTGCTCCT-3′*; MT-II forward *5′-TCCTGTGCCTCCGATGGATC-3′*; MT-II reverse 5′-GTCGGAAGCCTCTTTGCAGA-3′; MT-II taqman probe *5′-AAAGCTGCTGCTCCTGCTGCCCC-3′*; LCA1 (leukocyte common antigen 1) forward *5′-ACCTACATTGGAATTGATGCCATGCT-3′*; LCA1 reverse *5′-CCTCCACTTGCACCATCAGACA-3′*; LCA1 taqman probe *5′-GAAGCAGAGGGCAAAGTGGATGTCTATGG-3′*. All QRT-PCR reactions were carried out on an Applied Biosystems 7700 Taqman instrument (Applied Biosystems, Foster City, CA) in single tubes. The QRT-PCR reaction mix was composed of 1× PCR buffer (Invitrogen), 6 mM $MgCl_2$, 0.67 mM each dinucleotide, 6.75 pmols each of forward and reverse primers, 1.5 units of Superase-in (Ambion, Austin, TX), 15 units of Superscript II (Invitrogen), 0.75 units of Platininum *Taq* Polymerase (Invitrogen) to a final volume of 15 μl. Each reaction was run one cycle of 30′ at 42°, 1 cycle of 3′ at 95°, and 40 cycles of 1′ at 60° and 20′′ at 95°. QRT-PCR for the normalization gene, mouse LCA1, was performed as above but using LCA1 primers and probe set. Universal mouse RNA (Stratagene, La Jolla CA) was used to create a standard curve for each gene of interest and for the normalization gene, LCA1. Gene reactions were run in triplicate, averaged, and normalized to LCA1.

Lactate Dehydrogenase Assay for Determination of Cellular Toxicity

Global cellular toxicity was assessed using the Cyto-Tox lactate dehydrogenase assay (LDH) (Promega, Madison, WI). Briefly, after experimental treatment, 50 μl of medium was removed from each culture well and placed in a separate well-plate. Then, 50 μl of LDH assay solution was added to each well and allowed to incubate in the dark at room temperature for 30 min. At the end of the incubation period, 50 μl of stop solution was added,

and color change was measured at 490 nm on a Bio-TEK μQuant UV/Vis plate reader (BioTEK Instruments, Winooski VT). Net color change was determined by subtracting the average value of media-only wells from experimental values.

Lactate dehydrogenase release data were first normalized using the equation ([LDH $value_{Individual\ well}$]/[Control $Average_{Experiment}$]) to compare results between different experiments. Values for each condition were further transformed by calculating average percent level of protection compared to control using the equation: 100 – (100 × [Experimental/Control]).

MAP2/GFAP Colorimetric Assays for Determination of Cellular Toxicity

Microtubule-associated protein-2 (MAP2) and glia fibrillary–associated protein (GFAP) colorimetric assays were performed by following a modified protocol described by Carrier *et al.*[21] Briefly, treated and control cultures were washed 3 times in phosphate-buffered saline (PBS) and then fixed at room temperature in 4% paraformadehyde in 0.1% sodium phosphate (pH 7.4) for 30 min. Fixed cells were subsequently permeablized with 0.4% Triton-X 100 in PBS for 10 min at room temperature. Cells were then washed 3 times in PBS and blocked for 30 min in 10% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) at room temperature. Either rabbit anti-GFAP (sc-65343; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-MAP2 (sc-20172; Santa Cruz Biotechnology, Inc.) primary antibody in 3% bovine serum albumin/0.4% Triton-×100 in PBS (1:100) was added to fixed cultures and allowed to hybridize overnight at 4°C. Fixed cells were then washed 3 times in 0.5% Tween/PBS and hybridized for 2 h at room temperature with horse radish peroxidase- conjugated 2° antibody at 1:2000 (sc-2004; Santa Cruz Biotechnology, Inc.) in 1% bovine serum albumin/0.4% Triton-X 100 in PBS. Cell survival was monitored by measuring the horseradish peroxidase-catalyzed conversion of Amplex Red to resorufin (Invitrogen) with 1 mM H_2O_2 (1 mM; Sigma) in 50 mM phosphate buffer. Samples from each well were transferred to a 96-well plate, and absorbance (at 565 nm) was measured on a Bio-TEK μQuant UV/Vis plate reader.

MAP2, GFAP, MT-I/II Immunofluorescence Microscopy for Determination of MT Localization

Cells for localization studies were grown on polylysine-coated glass coverslips. Preparation for labeling was performed as described for colorimetric assays until the secondary labeling stage. Cultures were labeled with primary antibodies for MAP2 (rabbit anti-MAP2 polyclonal; sc-20172; Santa Cruz Biotechnology, Inc.), GFAP (rabbit anti-GFAP polyclonal; ab16997-1; AbCam), and MT-I/II (mouse anti-MT-I/II monoclonal; E-9; Zymed). The E9 clone has been demonstrated to be specific for MT-I/II and not to label other MT.[22] Secondary labeling was performed using fluorescent dye-conjugated 2°C antibodies (Alexa 488 goat antirabbit and Alexa 594 goat antimouse; Invitrogen; 1:4000) in 1% bovine serum albumin/0.4% Triton X-100 in PBS. Nuclei were counterstained for 3 min with 1× Hoechst (Invitrogen). Coverslips were mounted on slides and visualized using an Olympus BX51 epifluorescence microscope (Olympus America Inc, Center Valley, PA).

siRNA Knockdown of MT-I/II

A Dicer-substrate duplex that targets both MT-I and MT-II and sham duplex (designed not to target any genes) were obtained from Integrated DNA Technologies, Inc. RNA sequences for the MT-I/II knockdown duplex strands were *AGAACUCUUCAAACCGAUCUCUCGT* and *ACGAGAGAUCGGUUUGAAGAGUUCUAG*. Sequences for the scrambled sham duplex were not available from Integrated DNA Technologies. Cultures 10–12 days *in vitro* were transfected with siRNA constructs following instructions for use of TransPass R1 transfection reagent (New England Biolabs, Ipswich, MA). In brief, for each well, 400 μl of high-glucose serum-free DMEM (Invitrogen) was mixed for 20 min with 1.0 μl of transfection solution A, 2.0 μl of transfection solution B, and 20 μM duplex RNA to bring the final siRNA concentration to 5–10 nM. Cells were then washed 4 times with serum-free high-glucose DMEM (Invitrogen). Transfection mixture was added and cultures were returned to the incubator. After 2 h, the transfection mixture was removed and replaced with standard media. Cells were allowed to recover for 72 h before further treatment. Transfection efficiency was assessed using immunofluorescence microscopy to visualize the transfection of a fluorescein-labeled siRNA control (15 nM, Fluorescein-siRNA Transfection Control; New England Biolabs). siRNA knockdown of MT-I/II was assessed by QRT-PCR under both basal and MT-stimulating conditions (20 μM $ZnCl_2$, 12 h before harvest).[23,24] Cells were treated with sham siRNA for all control experiments.

MT-I/II Protein Transfection

Cells were transfected with either exogenous MT-I/II purified from horse liver (Sigma) or with a similarly sized control protein (insulin-oxidized ß-chain, Sigma) using ProteoJuice transfection reagent (Novagen, San Diego, CA). Briefly, transfection reagent and protein were mixed 1:2 (v:w) in 25 μl of serum-free DMEM and incubated at room temperature for 20 min. Meanwhile, cells were washed 4 times with serum-free DMEM and left in serum-free DMEM. The transfection mixture was added to each well to achieve a final protein concentration of 1.6–3.2 μM, and cells were returned to the incubator at

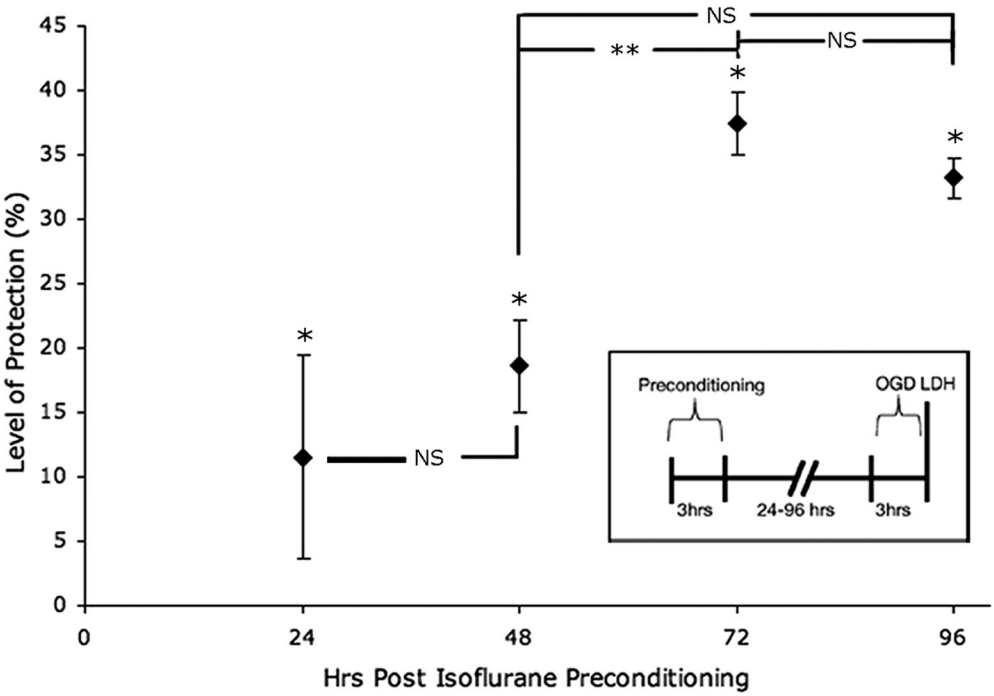

Fig. 1. Isoflurane induces delayed protection against oxygen glucose deprivation in murine mixed neuronal and glial cultures. Lactate dehydrogenase release (LDH) was used to assess oxygen-glucose deprivation (OGD)–mediated toxicity in both untreated and isoflurane-preconditioned (1.5% isoflurane; 3 h) cultures at 24, 48, 72, and 96 h after preconditioning. Percent protection (mean ± SEM) was calculated using the equation: 100 – (100 × ($Mean_{Test\ LDH}$/$Mean_{Control\ LDH}$)). Protection is reported as compared to days *in vitro*–matched controls. * = $P < 0.01$, two-tailed Student *t* test. Inset indicates timecourse for experimental procedure. ** = Significant difference between timepoints; *NS* = no significant difference for an ANOVA with Bonferroni *post hoc* analysis ($P < 0.05$).

37°C for 2 h. Transfected cells were washed 4 times in no-glucose DMEM and immediately placed in an anoxic chamber for OGD treatment. OGD toxicity was assessed by LDH.

Statistics

All statistics were performed in SPSS 9.0 (SPSS Inc, Chicago IL). ANOVA analyses were applied to data in figures 1, 2, and 3, with Bonferroni *post hoc* analyses where applicable (fig. 1). Figure 4 required no statistics. Figure 5 was analyzed by ANOVA. Two-tailed Student *t* test was applied to the data in figures 1, 3, 5, 6, and 7. Significance level was set at $P < 0.05$ unless stated otherwise.

Results

Isoflurane Induced Delayed Protection against Subsequent OGD-mediated Toxicity in Mouse Cortical Cultures

Isoflurane preconditioning has been shown to protect against ischemic injury in rat brain, providing improved histologic and neurologic outcomes 1 month after preconditioning/ischemia[7] and in neuron-enriched cortical cultures 24 h after isoflurane preconditioning.[2] To determine if isoflurane preconditioning protected over an extended period in culture conditions, we evaluated OGD toxicity in mixed cortical cultures at 24, 48, 72, and 96 h post preconditioning (fig. 1). A 3-h pretreatment with 1.5% v/v isoflurane (a clinically relevant exposure) provided protection that was significant (two-tailed Student *t* test; $P < 0.01$) compared to paired, nonpreconditioned controls for all time points. In detail, the protection at 24 h amounted to 11.6 ± 7.9% (n = 21), increasing to peak protection of 37.5 ± 2.5% at 72 h (n = 21). The effect persisted and was still robust at 96 h (33.4 ± 1.6%; n = 32). These data are consistent with isoflurane conferring delayed preconditioning *via* a direct pharmacological mechanism in dissociated primary cultures (see also Kapinya *et al.*[2]).

Isoflurane Preconditioning Protects Neurons and Glia against OGD Toxicity

To determine which cell lineages were protected against OGD by isoflurane preconditioning, we used an Amplex red-based immunoassay (fig. 2) to assess neuronal and glial survival after 3 h of OGD. Although 3 h of OGD was toxic to both neuronal and glial populations, neurons were particularly sensitive to this treatment, with survival rates of only 39.6 ± 8.6% *versus* 79.0 ± 4.1% (n = 16) for glial cells. Neurons were also more responsive to isoflurane preconditioning, showing recovery to a 84.9 ± 4.3% (n = 16) survival rate *i.e.*, approximately 127% increase in survival. To a

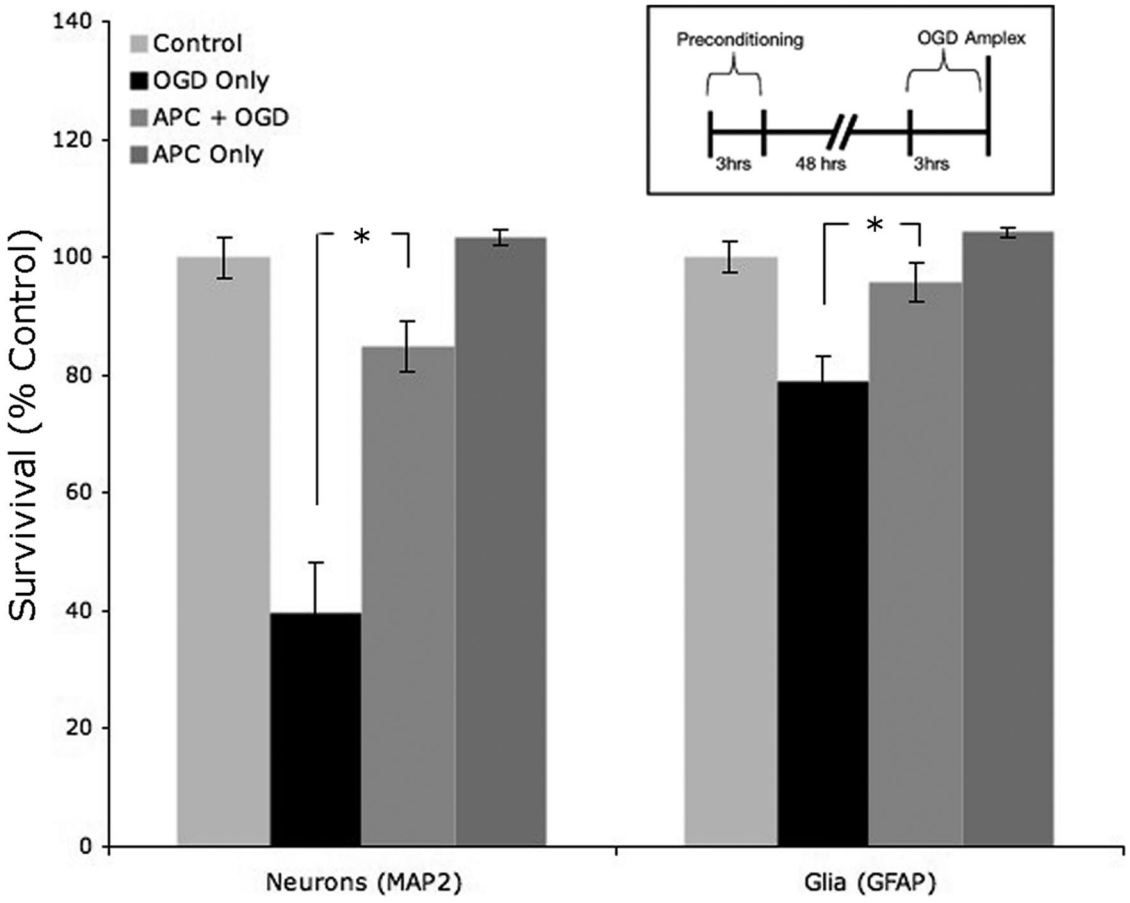

Fig. 2. Neurons and glia are protected by isoflurane preconditioning (APC). Mouse cortical cell culture subpopulations were assessed for oxygen-glucose deprivation (OGD)–induced toxicity and isoflurane-induced protection by immunolabeling for neurons with horseradish peroxidase–conjugated antibodies to microtuble–associated protein-2 (MAP2) and for glia with glial fibrillary–associated protein GFAP. * = $P < 0.01$, n = 16, two-tailed Student *t* test. Inset indicates timecourse for experimental procedure.

lesser extent, glial cells were also protected with a relative increase in survival rate of only approximately 22% (fig. 2). For neuronal populations, a 2 × 2 ANOVA analysis showed significant main effects of OGD and of isoflurane preconditioning (APC) with a significant interaction between these variables ($P < 0.01$). This interaction indicates the effectiveness of APC in counteracting OGD-mediated cell death and is supported by a Student *t* test that confirmed a significant ($P < 0.01$) difference between OGD alone and APC + OGD conditions (fig. 2). For glial cells, the 2 × 2 ANOVA analysis revealed significant main effects of OGD and of APC ($P < 0.05$); however, there was no significant interaction between these variables ($P = 0.53$). The lack of a significant interaction can be interpreted to mean that isoflurane preconditioning is not effective in protecting glia against OGD. However, a Student *t* test comparing the OGD alone and APC + OGD conditions revealed a significant ($P < 0.01$) difference between these groups, suggesting that APC was effective but also that the level of OGD toxicity was not as pronounced in this cell population.

MT-I/II Protein Transfection Provides Protection against OGD-mediated Toxicity

Previous studies have implicated MT-I/II in neuronal protection against ischemia (see Carmel *et al.*[17] and Wakida *et al.*[19]). Kennette *et al.*[25] determined that basal levels of MT were protective against certain toxins (*e.g.*, tert butyl peroxide); however, for other toxins (*e.g.*, cadmium and cisplatin) MT levels had to increase above basal levels for protection to occur. To discern in our system whether increased MT protein levels provided direct protection against OGD, we transfected cultures with exogenous MT-I/II protein before a 3-h OGD challenge and then assessed toxicity by LDH (fig. 3). Compared to transfection with a similarly-sized control protein (oxidized insulin β-chain), MT protein transfection conferred protection against 3 h of OGD, rising from 2.9 ± 2.0% protection at 1.6 μM MT (not significant; n = 8) to significant protection of 33.2 ± 3.1% ($P < 0.001$; n = 8) with 3.2 μM MT. In summary, these data confirm previous reports that MT are protective against ischemic toxicity in neuronal tissue.[26]

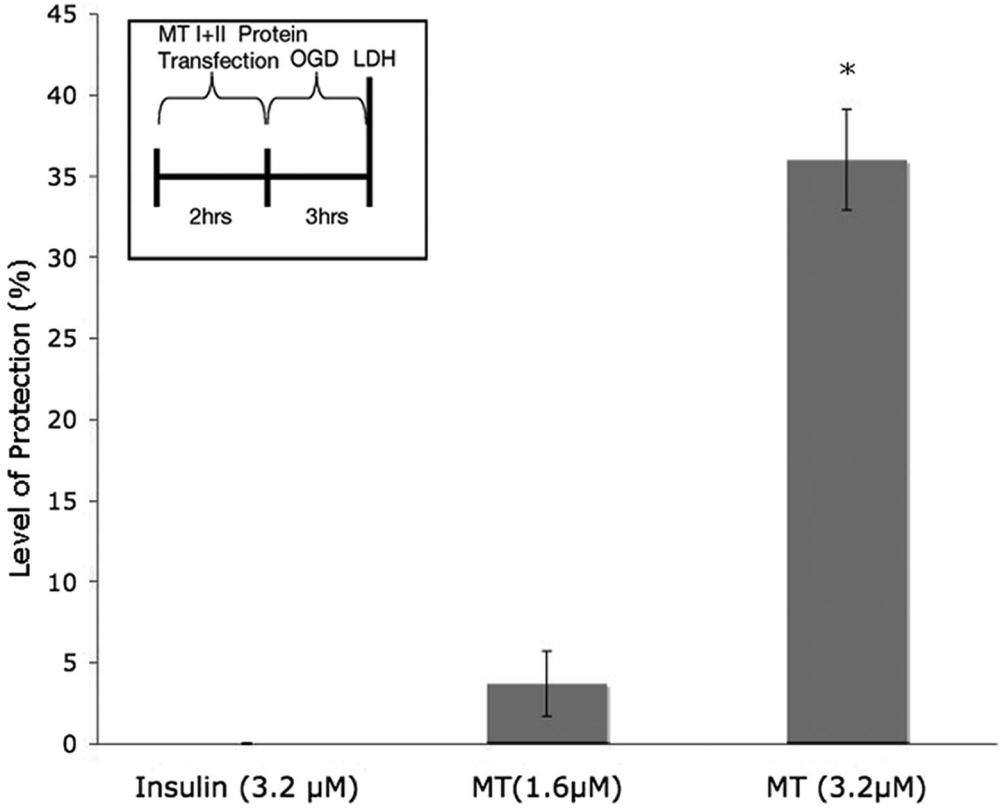

Fig. 3. Transfection with exogenous metallothionein-I/II (MT-I/II) protein protects primary cortical cultures against oxygen glucose deprivation (OGD)–mediated toxicity. Primary cortical cultures were transfected with 1.6 μM or 3.2 μM MT-I/II protein or 3.2 μM oxidized insulin β-chain (control). Toxicity was assessed by lactate dehydrogenase release assay (LDH). * = $P < 0.001$, two-tailed Student *t* test. Inset indicates time course for experimental procedure.

MT-I and MT-II Gene Expression Increased after Isoflurane-mediated Preconditioning

Given the precedent for regulation of MT gene expression after an ischemic episode[12] or an anesthetic exposure,[16] we examined whether MT-I and MT-II were induced in mouse mixed neuronal/glial after preconditioning with a 3-h 1.5% isoflurane exposure. Both MT-I and MT-II were rapidly upregulated, with increases in mRNA levels observed as early as 3 h (fig. 4). For MT-I/II, mRNA levels peaked at approximately 12 h, with levels that were 2.2 ± 0.2–fold and 2.8 ± 0.4–fold the nonpreconditioned cultures for MT-I and MT-II, respectively. Compared to untreated paired controls, MT-I mRNA fold changes were significant (two-tailed Student *t* test; $P < 0.05$) at 6 and 12 h, and MT-II mRNA fold changes were significant at 3, 6, and 12 h. These data are consistent with those described by Carmel *et al.* for ischemic-preconditioning of rat spinal cord[17] and indicates that MT are also regulated in response to isoflurane preconditioning. Although it would be preferable to establish the timecourse of the changes in protein levels post-APC, the small size and nature of the MT (*e.g.*, highly hydrophilic, number of cysteine residues) prevented us from resolving MT protein levels at the sensitivity required (see also Lu *et al.*).[27]

siRNA Knockdown of MT-I/II Enhances OGD-mediated Toxicity and Attenuates Isoflurane-induced Protection

To assess the protective role of MT-I/II in isoflurane preconditioning, we transfected cultures with either a sham siRNA construct or an siRNA construct that targeted both MT-I and MT-II before repeating the preconditioning/OGD regimen. We first determined the degree of MT-I and MT-II mRNA knockdown using QRT-PCR (fig. 5A). MT-I/II mRNA levels were assessed under both basal and MT-stimulating conditions (addition of 20 μM $ZnCl_2$, see Kim *et al.*[23]). Although we were able achieve only partial knockdown of either gene (fig. 5A), MT-I and MT-II mRNA levels were diminished in a dose-dependent manner by MT-siRNA transfection. Zinc stimulation and siRNA knockdown both showed greater effect on MT-II than on MT-I.

We assessed the effect of sham knockdown (ShamKD) or MT knockdown on isoflurane-mediated protection against 3-h OGD using an LDH assay (fig. 5B). Isoflurane-preconditioned ShamKD cultures demonstrated substantial protection (17.5 ± 5.2%, n = 27), similar to our findings for untreated cultures. ANOVA analysis of the siRNA data revealed main effects of MT knockdown ($P < 0.05$) and of APC ($P < 0.01$). However, there was no significant interaction found between these variables ($P = 0.518$).

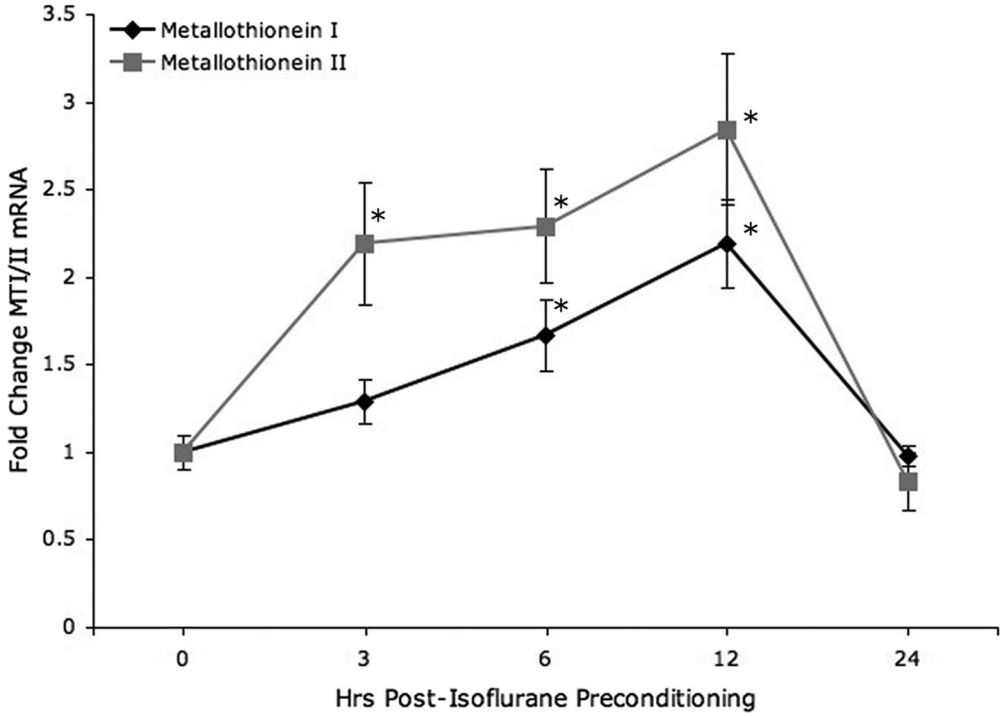

Fig. 4. Metallothionein I and II (MT-I/II) mRNA levels are increased after isoflurane preconditioning. Primary cortical cultures were preconditioned with 1.5% isoflurane for 3 h. Cells were collected at 5 time points (T = 0, 3, 6, 12, and 24 h) postexposure, and MT-I and MT-II mRNA levels were assessed by quantitative reverse trancriptase-polymerase chain reaction (QRT-PCR), normalized to leukocyte common antigen-1 (LCA1) expression. * = $P < 0.05$, n = 5, two-tailed Student *t* test).

This implies that the partial knockdown of the MT was not effective in reducing the protective influence of APC significantly. However, MT-I/II knockdown conditions without preconditioning showed substantially increased OGD-mediated toxicity compared to ShamKD (fig. 5B). This suggests that MT-I/II below typical basal levels renders cells particularly vulnerable to OGD insults. Due to the incomplete knockdown of MT-I/II in the siRNA experiments, we extended our study to perform APC/OGD experiments with MT-I/II knockout mouse–derived cultures.

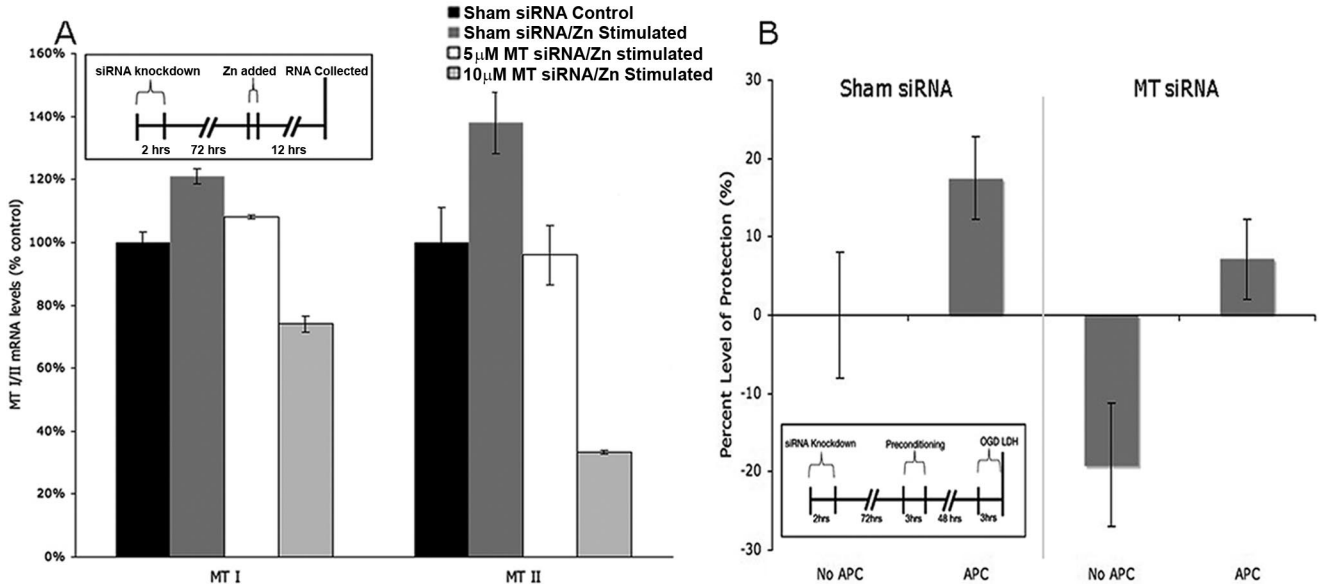

Fig. 5. Metallothionein short interfering RNA (siRNA) reduces metallothioneins I and II (MT-I/II) mRNA levels in a dose-dependent manner and increases vulnerability to oxygen glucose deprivation. (*A*) Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was used to assess MT-I/II knockdown by a siRNA against MT-I/II (MTKD). A sham siRNA (ShamKD) construct was used as the control. $ZnCl_2$ was used to simulate MT-stimulating conditions. (*B*) Oxygen-glucose deprivation (OGD)–mediated toxicity was assessed by lactate dehydrogenase release assay (LDH) in mixed cortical cultures transfected with either ShamKD or the MTKD siRNA constructs. See Results for discussion of significance (n = 27). Insets indicate time course for experimental procedure. APC = isoflurane preconditioning.

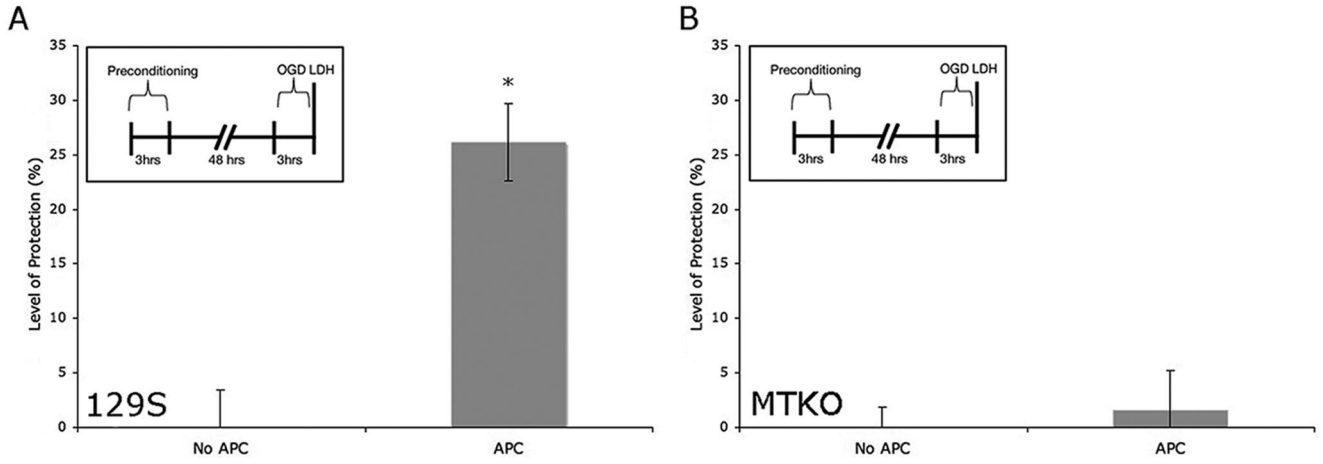

Fig. 6. Knockout of metallothionein-I/II (MT-I/II) genes abolishes isoflurane-mediated protection against oxygen-glucose deprivation. Oxygen glucose deprivation (OGD)–mediated toxicity was evaluated after 3-h 1.5% isoflurane preconditioning (APC) in mixed neuronal/glial cultures from (*A*) background 129 and (*B*) MT-I/II knockout mice (MTKO). * = $P < 0.001$, two-tailed Student *t* test. Inset indicates timecourse for experimental procedure. LDH = lactate dehydrogenzse release assay.

MT-I/II Knockout Abolishes Isoflurane-mediated Protection against OGD Toxicity

As a more definitive method of addressing the importance of MT to isoflurane preconditioning, we compared treatments on cultures prepared from MT-I/II knockout mice (see Materials and Methods) to cultures derived from the background 129 strain (fig. 6). Similar to our previous experiments with C57 mice (fig. 1), preconditioned background 129-derived cultures showed significant protection (two-tailed Student *t* test; $P < 0.001$) against OGD toxicity (26.2 ± 3.6%; n = 16) as compared to paired nonpreconditioned samples. By contrast knockout-derived cultures (MTKO) showed no protection from isoflurane preconditioning (1.5% ± 3.7%; n = 25, fig. 6B). Both 129 wild-type (WT) and MTKO cultures were assessed by LDH for toxicity from isoflurane preconditioning alone. Preconditioned 129 WT cultures showed LDH levels of 97 ± 11% of untreated cultures, and MTKO cultures showed LDH levels 124 ± 19% of their untreated counterparts at 48 h after isoflurane exposure (n = 16). There was no significant difference between treated and untreated cultures in either 129 WT or MTKO cultures (two-tailed Student *t* test, $P = 0.86$ and $P = 0.49$, respectively). These data provide compelling evidence of a critical role for MT in isoflurane-mediated preconditioning.

MT-I/II Proteins Are Primarily Localized to Neuronal Cytoplasm in Mouse Neonatal Cortical Cultures

In light of our finding that APC provided more protection from OGD to neurons rather than glia, we determined the cellular localization of MT-I/II proteins (fig. 7). Previous studies in mouse brain have shown MT-I/II to be localized primarily in glial cells.[19,28,29] Here we used immunofluorescence microscopy to assess the colocalization of a monoclonal MT-I/II antibody with either neuron-specific MAP2 antibodies or glial-specific GFAP antibodies. In 129-derived cells, the MT antibody colocalized strongly with MAP2-labeled cell bodies, indicating strong neuronal localization (fig. 7A). GFAP-labeled cells showed MT colocalization to a lesser extent (data not shown). Neither MT knockout-derived cultures nor 129 WT cultures coincubated with 10 μM horse MT-I/II protein demonstrated measurable MT labeling, consistent with MT-I/II–specific labeling (fig. 7, B and C).

Discussion

Delayed anesthetic preconditioning has been shown to be a powerful tool for improving both histologic and clinical outcomes against ischemia-mediated toxicity in a number of tissues.[12,30] Given the relative safety of pre-

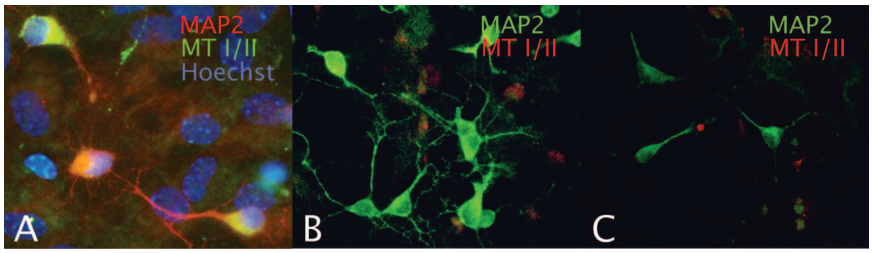

Fig. 7. Metallothioneins-I/II (MT-I/II) localize to neuronal cell bodies in cortical neuronal cultures. MT-I/II localization was visualized by epifluoresence microscopy. Cultures 10 days *in vitro* were labeled using a monoclonal antibody against MT-I/II and a polyclonal antibody against microtube-associated protein-2 (MAP2). Secondary labeling was accomplished with Alexafluor 488 or Alexafluor 594 fluorescent conjugates. (*A*) WT 129 cells, nuclei were stained with Hoechst. (*B*) MT-I/II knockout cells. (*C*) WT 129 cultures, antibody preincubated with 10 μM horse MT-I/II protein to show MT antibody specificity.

conditioning with anesthetics *versus* surgically induced ischemia,[31] there is emerging interest in anesthetic preconditioning. In this study, we used a dissociated cortical neuronal culture model to investigate the direct pharmacological effects of isoflurane preconditioning and the role that MT-I/II play in mediating that protection. An *in vitro* dissociated tissue culture model of neuronal APC/OGD presents a number of advantages over in *vivo models*, including the ability to measure direct pharmacological effects of the anesthetic agent while avoiding the confounding influences of hemodynamics, cardiac depression, and other anesthesia-mediated physiologic factors. An *in vitro* model also provides more flexibility for manipulation and direct observation of outcomes in studying molecular mechanisms. Inevitably, however, a dissociated culture model has limitations. For instance, the *in vitro* OGD model cannot be used to assess system-wide effects of ischemic injury such as inflammatory and immune response to injury. Furthermore, focal ischemia models enable observations of long-term outcomes *in vivo*. Despite these constraints, we have shown that dissociated culture with OGD is a useful model for studying APC and OGD and that the timecourse of protection mirrors that of *in vivo* models.[2,30] In the future, it will be necessary to follow-up our experiments with studies on the role of MT during anesthetic preconditioning *in vivo*. Moreover, as MT can be induced through various conditions,[32] this work may have important implications for improved clinical outcomes.

The principle findings of our study are as follows. (1) Pretreatment with 1.5% isoflurane provided robust protection against OGD toxicity that peaked 72 h after isoflurane exposure. (2) OGD was more toxic to neurons than to glia, although preconditioning with isoflurane significantly reduced toxicity in both populations. (3) Exogenous MT protein supplementation was directly protective against OGD toxicity. (4) MT-I/II mRNAs were rapidly and robustly induced by treatment with clinically relevant doses of isoflurane. (5) siRNA knockdown of MT-I/II– diminished isoflurane-mediated protection against an OGD insult. (6) MT-I/II gene knockout abolished isoflurane-mediated protection and rendered cultures more susceptible to OGD than WT cultures. (7) MT-I/II localized to neuronal cell bodies, although they were also seen in glial cytoplasm. The combination of these data argues for an important role of MT-I/II in isoflurane-mediated protection against OGD. To our knowledge, this is the first time MT-I/II have been revealed as important mediators of isoflurane preconditioning.

There are several reports supporting a protective role of MT-I/II in a number of tissues. In particular, MT-I/II have been implicated in protection against ischemic injury in brain,[19,33] heart,[18,34] and kidney.[35] Campagne *et al.*[33] found MT-overexpressing mice had not only better histologic outcomes but also significantly improved motor performance 3 weeks after focal cerebral ischemia/reperfusion. Wakida *et al.* [19] found erythropoietin-induced MT-I/II reduced infarct area and volume at 24 h after permanent middle carotid artery occlusion damage in rat brain. Of direct relevance to our study, Carmel *et al.*[17] found that MT-I/II mRNAs were rapidly induced in rat spinal cord after brief ischemic preconditioning. These data complement our findings that MT-I/II are rapidly induced after isoflurane preconditioning and that the increased MT levels provide protection against subsequent ischemic injury.

A surprising result was the localization of MT-I/II to neuronal cell bodies along with weaker glial staining (fig. 7). Although this localization correlates well with the robust APC-mediated neuronal protection (fig. 2), it contrasts with previous reports that MT-I/II localize primarily within glia in the brain with little to no expression in neurons.[28] These localization differences may be accounted for by a number of experimental variations, including developmental stage of our cells, differences in structure of cells grown in culture *versus in vivo*, or differences in fixing procedures (see Hidalgo *et al.*[28]). Neuronal localization may be accounted for by recent work by Chung *et al.*,[36] who found that MT-I/II can be exported by astrocytes and readily taken up and used by neurons.

Although our study demonstrated that MT-I/II can be protective against OGD, the mechanism by which MT-I/II confer this protection remains to be determined. MT are small (approximately 6000–7000 Da) metal-binding (primarily $Zn^{2+}$and Cu^{2+}) proteins that are expressed throughout the body. They exhibit a number of cellular activities that could potentially account for their protective role against ischemia (see Penkowa[32]). These roles include modulation of cellular redox, immune defense response, mitochondrial respiration, angiogenesis, cell-cycle progression, cell survival, and differentiation. An intriguing role for MT in preconditioning is as a critical component of the nitric oxide signaling cascade[37] that has been identified as essential for induction of delayed isoflurane-induced preconditioning in heart.[30] Stitt *et al.*,[38] studying nitric oxide signaling in mouse lung endothelial cells, showed that MTs were essential for nitric oxide-mediated translocation of the transcription factor metal-responsive transcription factor–1 to the nucleus and for subsequent upregulation of MT. These data support a role for MTs in nitric oxide signaling during ischemic stress.

In addition to possibly participating in nitric oxide signaling, another potential role for MT in APC-mediated protection is as an end effector of cellular protection. This is supported by the findings shown in figure 3, in which transfection of exogenous MT-I/II protein provided immediate protection against OGD damage. As an end effector, MT might act as antioxidants to detoxify reactive oxygen species produced by the energy pathway imbalances of ischemia (see Penkowa[32]). In support

of this, increased MT levels have been shown to decrease both reactive oxygen species levels and peroxidation of cellular proteins and lipids.[39] Furthermore the antioxidant effects of MT during mitochondria-specific oxidative stress have been shown to result in greater protection than provided by Cu/Zn-SOD, glutathione peroxidase, Mn-SOD, or catalase.[40] Alternatively, MT-mediated protection may depend on the central role MTs play in zinc homeostasis.[32] Approximately 3% of the mammalian proteome requires zinc for some aspect of functionality, with 40% of zinc-binding proteins acting as transcription factors and the remaining 60% of proteins involved in ion transport or as enzymes.[41] Through their role in zinc homeostasis, MT may have pleiotropic effects in ischemic tissue, which affect a range of responses from transcription to ion homeostasis through modulation of zinc availability throughout the cell.

In conclusion, this study describes an important role for MT-I/II in isoflurane preconditioning against OGD. On the basis of the role that MT play in isoflurane-mediated delayed protection against OGD, our results implicate MT-I/II as possible molecular targets for improving clinical outcomes during ischemic injury.

The authors thank the Blakeslee and Wilens Funds of Smith College (Northampton, Massachusetts) for financial support of this project. They also thank Smith College students Eva LaDow, B.A., Jeanne McKeon, B.A., Emily Stackpole, B.A., Kristen Hokenson, B.A., Laura-Louise Campbell, B.A., and Jennifer Tyson, B.A., for support with the tissue culture. Finally, the authors thank the Animal Care Facility at Smith College (Joanne Huyler, D.V.M., Director, Donna Ewell, Animal Technician, Vickie Flood, Animal Technician) for mouse breeding and animal care.

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