Trauma-Induced Cell Swelling in Cultured Astrocytes

Arunugam R. Jayakumar, PhD, K.V. Rama Rao, PhD, Kiran S. Panickar, PhD, M. Moriyama, PhD, P.V.B. Reddy, PhD, and Michael D. Norenberg, MD

Abstract

Brain edema and associated increased intracranial pressure are major consequences of traumatic brain injury that account for most early deaths after traumatic brain injury. An important component of brain edema after traumatic brain injury is astrocyte swelling (cytotoxic edema). To examine the pathophysiologic mechanisms of trauma-induced astrocyte swelling, we used an in vitro fluid percussion trauma model. Exposure of cultured rat astrocytes to 5 atm of pressure resulted in significant cell swelling at 1 to 24 hours posttrauma that was maximal at 3 hours. Because oxidative/nitrosative stress, mitochondrial permeability transition (mPT), and mitogen-activated protein kinases (MAPKs) have been implicated in astrocyte swelling in other neurologic conditions, we examined their potential roles in this model. We previously showed increased free radical generation after in vitro trauma and show here that trauma to astrocytes increased the production of nitric oxide. Trauma also induced mPT and increased phosphorylation (activation) of MAPKs (extracellular signal-regulated kinase 1/2, c-Jun-N-terminal kinase, and p38-MAPK); these changes were diminished by antioxidants and the nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester. Antioxidants, N-nitro-l-arginine methyl ester, the mPT inhibitor cyclosporin A, and inhibitors of MAPKs all significantly diminished trauma-induced astrocyte swelling. These findings demonstrate that direct mechanical injury to cultured astrocytes brings about cell swelling, and that blockade of oxidative/nitrosative stress, mPT, and MAPKs significantly reduce such swelling.

Key Words: Astrocytes, Cell cultures, Cell swelling, Cytotoxic edema, Mitochondrial permeability transition, Mitogen-activated protein kinases, Nitric oxide, Oxidative stress, Traumatic brain injury.

INTRODUCTION

Brain edema is a major neurologic complication of traumatic brain injury (TBI). A consequence of edema is the development of increased intracranial pressure leading to brain herniation and brainstem compression. The edema and its associated complications account for approximately 50% of deaths in patients with TBI (1). Although both vasogenic and cytotoxic mechanisms contribute to TBI-related brain edema (2), cytotoxic edema (intracellular swelling) predominates in the early phase (2–24 hours) of TBI (3, 4), with astrocytes being the cells that are principally affected (5). Treatment options for cytotoxic edema after trauma are currently limited and are, for the most part, not effective (6).

Mechanisms responsible for trauma-induced cytotoxic edema are poorly understood, but a number of factors have been implicated in TBI-mediated astrocyte swelling. These include elevated extracellular K⁺ and intracellular Ca²⁺, tissue acidosis, free fatty acids (including arachidonic acid), and the release of excitatory amino acids (3, 6–8).

Several factors are known to be involved in the mechanism of injury after central nervous system (CNS) trauma and in the evolution of brain edema in other neurologic conditions. These include oxidative/nitrosative stress (ONS) (9, 10), the activation of mitogen-activated protein kinases (MAPKs) (11), and the mitochondrial permeability transition (mPT) (12, 13). Accordingly, this study examined the potential involvement of these factors in the pathogenesis of trauma-related astrocyte swelling.

Because the cellular complexity of the CNS precludes a comprehensive analysis of individual cell responses to injury, we investigated mechanisms of trauma-induced astrocyte swelling using a cell culture approach. Our findings indicate that trauma to astrocytes causes cell swelling; the swelling is associated with ONS, the mPT, and MAPK activation. Moreover, inhibition of these processes results in a marked attenuation of trauma-induced astrocyte swelling. A preliminary account of these findings has been presented (14).

MATERIALS AND METHODS

Astrocyte Cultures

Astrocyte cultures were prepared from brains of 1-day-old rat pups by the method of Ducis et al (15). Briefly, cerebral cortices were freed of meninges; minced; dissociated by trituration and vortexing; passed through sterile nylon sieves; placed in Dulbecco’s modified Eagle medium (Life Technologies, Gaithersburg, MD) containing penicillin, streptomycin, and fetal bovine serum; and incubated at
37°C in a humidified incubator provided with 5% CO₂ and 95% air. After 10 days in culture, bovine serum was replaced with 10% horse serum. After 14 days, cultures were treated and maintained with dibutyryl cyclic adenosine monophosphate (Sigma, St. Louis, MO) to enhance cell differentiation (16). Cultures consisted of at least 98% astrocytes as determined by glial fibrillary acidic protein and glutamine synthetase immunocytochemistry. The remaining cells consisted of microglia. Experiments were carried out in 3- to 4-week-old cultures. Procedures followed guidelines established by the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the University of Miami Institutional Animal Care and Use Committee.

In Vitro Trauma

Trauma was induced in cultured astrocytes using a fluid percussion device first developed by Sullivan et al (17) and modified for cell culture by Shepard et al (18). The injury chamber was coupled to the fluid percussion device with nondistensible Tygon tubing, and the piston was percussed by the weighted pendulum at varying angles of incidence. Five atmospheres of pressure were administered twice for a 25-millisecond duration each. Pressures were continuously recorded with a PowerLab system (ADInstruments, Inc., Colorado Springs, CO) interfaced with a high-speed pressure transducer. Sham controls were treated exactly like the traumatized cells, except that fluid percussion was not performed.

Cell Volume Determination

Cell volume was estimated by measuring the intracellular water space by the method of Kletzien et al (19), as modified for cell cultures by Kimelberg (20) and Norenberg et al (21). Briefly, 1 mmol/L of 3-O-methylglucose and 0.5 µCi/ml [3H]-3-O-methylglucose (Sigma) were added to the culture 6 hours before the volume assay. At the end of the incubation period, culture medium was aspirated, and an aliquot was saved for radioactivity determination. Cells were rapidly washed 6 times with ice-cold buffer containing 229 mmol/L of sucrose, 1 mmol/L of Tris-nitrate, 0.5 mmol/L of calcium nitrate, and 0.1 mmol/L of phloretin, pH 7.4. Cells were harvested into 0.5 ml of 1N sodium hydroxide. Radioactivity in the cell extracts and medium was determined, and an aliquot of the cell extract was used for protein estimation by bicinchoninic acid method. Values were normalized to protein level, and cell volume was expressed as a percent change over sham control. Cell volume in sham controls was 2.4 ± 0.17 µl/mg protein.

Measurement of Nitric Oxide

Nitric oxide (NO) production was analyzed in cultured astrocytes using DAF-2DA, a green fluorescence probe specific for intracellular NO (22). Briefly, sham control and traumatized culture plates were washed 3 times with serum-free culture medium and incubated with DAF-2DA (Molecular Probes, Eugene, OR; 10 µmol/L) for 15 minutes at 37°C. Cultures were then washed 2 to 3 times with phosphate-buffered saline (PBS), and cells were scraped into 500 µl of 0.2% Triton X-100 and sonicated for 5 seconds. A small aliquot of the cell extract was removed for protein estimation, and 250 µl of the extract was transferred to a 96-well microtiter plate (Dynex Technologies Inc, Chantilly, VA). Fluorescence was measured at an excitation wavelength of 492 nm and an emission wavelength of 515 nm. The results were expressed as fluorescence intensity units per milligram of protein.

Measurement of the Mitochondrial Inner Membrane Potential

The mitochondrial inner membrane potential (ΔΨₘ) was measured with the fluorescent dye tetramethylrhodamine ethyl ester (TMRE; Invitrogen, Carlsbad, CA), as previously described (23). At different points after trauma, astrocytes were loaded with TMRE to a final concentration of 50 nmol/L for 20 minutes. The cells were then examined with a Nikon Diaphot inverted fluorescent microscope equipped with multivariate fluorescent filters. The fluorescence emission was recorded at 590 nm. To avoid mitochondrial leakage of TMRE due to photobleaching, the cells were exposed to low fluorescent light intensity. Fluorescent intensities were then analyzed using the Sigma Scan Pro quantitation software (Jandell Scientific, San Jose, CA). The total number of pixels was quantified on a gray scale (0–255), and the average pixel (fluorescent intensity) value in each image, containing an approximately equal number of cells, was obtained and expressed as the mean ± SEM of the total fluorescence intensity derived from a minimum of 15 random (20×) fields. The selection of fields was achieved by systematically moving the microscope stage by 5 mm² in all 4 directions. The investigator was “blinded” as to whether control or traumatized plates were being examined. Arbitrary TMRE fluorescent intensities were converted to percentages and expressed as a percent fluorescence change over sham control.

To exclude the possibility that potential changes in the plasma membrane may have influenced TMRE loading, astrocyte cultures were exposed to different concentrations (10–100 mmol/L) of KCl for 30 to 45 minutes to depolarize the plasma membrane. Untreated and KCl-treated cells were then incubated with 50 nmol/L of TMRE (20 minutes). KCl had no effect on mitochondrial TMRE fluorescence levels in astrocytes as compared with untreated cells, indicating that changes in the plasma membrane potential did not interfere with ΔΨₘ measurements.

Mitochondrial Permeability of 2-Deoxyglucose-6-Phosphate

As another method for measuring the mPT, we used an adaptation of the in vivo 2-deoxyglucose (2DG) entrapment method of Kerr et al (24), as modified for cell cultures by Bai et al (25), which measures changes in mitochondrial membrane permeability. This method is based on the principle that [3H]-2DG enters the cell via the glucose transporter present on the plasma membrane and is then phosphorylated by hexokinase to [3H]-2DG-6-phosphate (2-DG-6-P). Because the inner membrane of normal mitochondria is not permeable to [3H]-2DG-6-P, very little label is found in
mitochondria of normal cells. When the mPT is induced, however, \(^{1}H\)-2-DG-6-P is able to enter mitochondria through the permeability transition pore, which then equilibrates with the cytosolic pool of the isotope. Closure of the permeability transition pore at this stage with a Ca\(^{2+}\) chelator (EDTA) results in the entrapment of \(^{1}H\)-2-DG-6-P. The presence of labeled \(^{1}H\)-2-DG-6-P in mitochondria indicates that mitochondria have undergone the mPT (24).

The mitochondrial preparation used in this assay employed the permeabilized cell procedure of Huang and Philbert (26) as modified by Bai et al (25). The medium was aspirated, and the cells were washed 3 times with ice-cold PBS. To each culture dish, 0.25 ml of ice-cold isolation buffer (250 mmol/L of mannitol, 2.5 mmol/L of EDTA, and 17 mmol/L of 3-[N-morpholino]propane sulfonic acid; pH 7.4) was added, and the culture plate was kept on ice for 5 minutes. Cells were harvested in the same medium containing an ice-cold solution of digitonin (0.1 mg/ml) to solubilize the plasma membrane. After 1 minute, the tubes were centrifuged at 14,000 \(\times\) g for 1 minute. The supernatant was discarded, and the pellet was washed twice in a large volume of ice-cold medium and resuspended in isolation buffer (40 \(\mu\)l per plate).

Sham control and traumatized astrocyte cultures (both at 3 and 24 hours after trauma) were incubated with 1 mmol/L of 2DG dissolved in medium containing 0.5 \(\mu\)Ci \(^{1}H\)-2DG (Perkin Elmer, Waltham, MA) for 1 hour at 37°C. At the end of incubation, an aliquot of medium from each culture plate was collected to determine the total amount of radioactivity. Plates were washed 3 times with ice-cold PBS and then permeabilized with digitonin, as previously described. Permeabilized cells completely devoid of cytosol were resuspended in a small volume of isolation buffer, and an aliquot of the cell suspension was transferred to scintillation vials for radioactivity determination, whereas the rest of the sample was retained for determination of citrate synthase to correct for mitochondrial recovery. Citrate synthase activity was determined following the method of Shepherd and Garland (27). The disintegrations per minute values in the sham control group were set at 100%. The 2-DG-6-P entrapment method has been validated in our laboratory for use in cell cultures by using known inducers of the mPT such as 1-butylhydroperoxide and phenylarsine oxide (25).

### Measurement of Adenosine Triphosphate

Cultured astrocytes 3 hours after trauma were quickly washed in ice-cold PBS, rapidly frozen, harvested into 12% trichloroacetic acid, and centrifuged at 11,750 \(\times\) g. The clear supernatant was used for the estimation of adenosine triphosphate (ATP) using the Sigma Diagnostic Kit (catalog no. 366-UV) based on the method of Adams (28), whereas the pellet was used for protein determination. Briefly, 0.5 ml of cell extract was added to a total reaction mixture volume of 3.3 ml containing 1 ml of phosphoglyceric acid buffer (18 mmol/L), 1 mmol/L of magnesium, 1 mmol/L of EDTA, 1.5 ml of water, and 0.3 ml of nicotinamide adenine dinucleotide (reduced form) (1 mg/ml), and the reaction was initiated by the addition of a glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase enzyme mixture in a 2:1 ratio. The decrease in the absorbance due to the oxidation of nicotinamide adenine dinucleotide (reduced form) was followed for 6 minutes, and the change in absorbance was corrected for protein content.

### Western Blots

Astrocyte cultures were solubilized in lysis buffer (125 mmol/L of Tris-HCl, pH 6.8), 4% sodium dodecyl sulfate, phosphatase inhibitors, and protease inhibitor mixture (Roche Biosciences, Palo Alto, CA), and protein levels were measured by the bicinchoninic acid method. Equal amounts of protein were subjected to gel electrophoresis as previously described (11) and electrophoretically transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk, membranes were incubated with respective antibodies. Primary antibodies to detect phosphorylated forms of extracellular signal-regulated kinase (ERK)1/2, c-Jun-N-terminal kinase (JNK), and p38-MAPK were used at 1:1000 (Cell Signaling Technology, Danvers, MA). Anti-\(\alpha\)-tubulin antibody was obtained from Oncogene Science (Cambridge, MA). Anti-rabbit and anti-mouse (Vector Laboratories, Burlingame, CA) horseradish-peroxidase-conjugated secondary antibodies were used at 1:1000. Optical density of the bands was measured with the Chemi-Imager (Alpha Innotech, San Leandro, CA) digital imaging system, and the results were quantified with the Sigma Scan Pro software as a proportion of the signal of a housekeeping protein band (\(\alpha\)-tubulin).

### Statistical Analysis

Each group consisted of 4 to 5 culture dishes per experiment for each point studied. At least 2 to 4 plates were used for Western blot analysis. Experiments were performed in cultures taken from 4 to 7 separate seedings. Data were subjected to analysis of variance, followed by Tukey post hoc comparisons. Intensity unit values obtained from optical density of the bands in Western blots were also subjected to statistical analysis as previously described. At each point, the experimental cultures were compared with their respective sham controls.

### RESULTS

#### In Vitro Trauma Causes Astrocyte Swelling

Cells were traumatized, and cell volumes were determined after different periods. Astrocyte swelling was significantly increased at 1 to 24 hours posttrauma, with maximal swelling occurring at 3 hours (55%; \(p < 0.05\) vs sham control; Fig. 1). Although cell swelling was documented by an analytic method, no significant cytologic changes were detected in Giemsa-stained preparations examined by light microscopy (data not shown).

#### Trauma Results in NO Production

We previously showed that trauma caused a significant increase in reactive oxygen species (ROS) generation in astrocyte cultures (29). In the present study, a significant increase in NO level was observed at 20 minutes; levels peaked at 1 hour and remained elevated for at least 6 hours (Fig. 2).
Antioxidants and \( N \)-Nitro-L-Arginine Methyl Ester Diminish Trauma-Induced Astrocyte Swelling

Ten minutes prior to trauma, cells were treated with antioxidants, including superoxide dismutase (SOD; 20 U/ml), catalase (500 U/ml), vitamin E (150 \( \mu \)mol/L), or \( N \)-tert-butyl-\( \alpha \)-phenylnitrone (PBN; 100 \( \mu \)mol/L), or the NO synthase inhibitor \( N \)-nitro-L-arginine methyl ester (L-NAME; 250 \( \mu \)mol/L). The effects of these agents on cell swelling were measured 3 hours after trauma (i.e. the time of maximal swelling). Trauma increased cell swelling by 53% compared with sham controls (\( p < 0.05 \)) (Fig. 3). Vitamin E, catalase, and PBN significantly diminished cell swelling by 79%, 56%, and 75%, respectively (\( p < 0.05 \) vs trauma for each; Fig. 3). \( N \)-Nitro-L-arginine methyl ester also decreased trauma-induced swelling by 53% (\( p < 0.05 \) vs trauma). Superoxide dismutase also attenuated the trauma-induced cell swelling, but the decrease was not statistically significant (Fig. 3). Cultures treated with the previously discussed antioxidants and L-NAME 10 minutes after trauma also showed a similar degree of inhibition of cell swelling (data not shown). Cultures treated with the same volume of solvents or with antioxidants and L-NAME alone, in the absence of trauma, did not influence cell volume. The concentrations of agents used in these experiments had been found to be most effective in dose-response studies.

In Vitro Trauma Induces the mPT

A decline in the \( \Delta \Psi_m \) (27%; \( p < 0.05 \)) was observed as early as 10 minutes after trauma (i.e. the earliest time it can be measured; the greatest degree of dissipation was at 1 hour (45%), persisting for up to 3 hours. Treatment of astrocytes 10 minutes before trauma with the mPT inhibitor cyclosporin A (CsA; 1 \( \mu \)mol/L) significantly blocked the loss of the \( \Delta \Psi_m \) at all points (Figs. 4 and 5). To rule out the possibility that the effect of CsA was due to its calcineurin blocking effect, rather than its mPT inhibitory action, we examined the effect of FK506 (1 \( \mu \)mol/L; Fujisawa USA, Deerfield, IL). FK506, similar to CsA, has calcineurin inhibitory effects but has no influence on the mPT. FK506 did not block the trauma-induced dissipation of the \( \Delta \Psi_m \) (data not shown).

Determination of the mPT by the 2-DG-6-P permeability method showed a 125% increase in 2-DG-6-P permeability 3 hours after trauma compared with sham controls (\( p < 0.01 \)). Ten-minute pretreatment with CsA (1 \( \mu \)mol/L) completely blocked this effect (Fig. 6). On the other hand, FK506 did not affect 2-DG-6-P permeability. These data, in conjunction with the results of changes in TMRE fluorescence (Figs. 4 and 5), indicate that in vitro trauma induces the mPT in cultured astrocytes.

In Vitro Trauma Reduces ATP Levels in Astrocytes

At 3 hours after trauma cellular ATP levels were reduced (27%; \( p < 0.05 \)), this reduction was completely blocked by CsA (1 \( \mu \)mol/L). Sham control ATP levels were 65 ± 5 nmol/mg protein.
Antioxidants and L-NAME Diminish Trauma-Induced mPT

Astrocytes were treated with SOD (20 U/ml), catalase (500 U/ml), vitamin E (150 μmol/L), and L-NAME (250 μmol/L) 10 minutes before trauma, and changes in the TMRE fluorescence were determined 3 hours after trauma (time of maximal mPT). Trauma dissipated the $\Delta\Psi_m$ by 35% as compared with sham controls ($p < 0.05$), and such dissipation was significantly blocked by SOD (81%), vitamin E (67%), and L-NAME (76%), whereas catalase had no effect (Fig. 7).

FIGURE 5. Quantification of the time course of the cyclosporin A (CsA)-sensitive decrease in the $\Delta\Psi_m$ by trauma in astrocytes. The average arbitrary tetramethylrhodamine ethyl ester (TMRE) fluorescence intensities in each experimental group were converted to percentages. *, $p < 0.05$ versus sham and $\dagger$, $p < 0.01$ versus trauma.

FIGURE 6. Effect of trauma on the mitochondrial permeability transition as measured by $[^3H]$-2-deoxy-glucose-6-phosphate (2-DG-6-P) entrapment method in cultured astrocytes. Values are expressed as percent disintegrations per minute (DPM) of 2-DG-6-P per unit citrate synthase. Trauma (T) at 3 hours caused a 125% increase in 2-DG-6-P permeability, which was blocked by cyclosporin A (CsA) (1 μmol/L). FK506 had no significant effect. *, $p < 0.01$ versus sham control; $\dagger$, $p < 0.01$ versus trauma. C, control.

FIGURE 7. Quantification of the effect of antioxidants and N-nitro-L-arginine methyl ester on the trauma-induced dissipation of the $\Delta\Psi_m$. The average arbitrary tetramethylrhodamine ethyl ester (TMRE) fluorescence intensities in each experimental group were converted to percentages. *, $p < 0.05$ versus sham control; $\dagger$, $p < 0.01$ versus trauma. C, control; CAT, catalase; LN, N-nitro-L-arginine methyl ester; SOD, superoxide dismutase; Vit E, vitamin E.
Inhibition of the mPT Attenuates Trauma-Induced Astrocyte Swelling

To investigate the potential role of the mPT in cell swelling, we examined the effect of CsA on trauma-induced astrocyte swelling. Astrocytes exposed to trauma increased cell volume at 3 hours by 56% as compared with sham controls. A 10-minute pretreatment with CsA (1 μmol/L) significantly diminished cell swelling by 72% (Fig. 8), although treatment with CsA 10 minutes after trauma did not block trauma-induced astrocyte swelling (data not shown). In contrast to CsA, FK506 did not block astrocyte swelling (Fig. 8).

In Vitro Trauma Activates MAPKs

Astrocytes were exposed to trauma, and MAPK phosphorylation (activation) was determined at different periods by Western blot. Thirty minutes after trauma, there was a 3-fold increase in phosphorylated ERK1/2 and JNK1. This increase remained elevated for up to 3 hours (Fig. 9). A slight, but significant, increase in p38-MAPK phosphorylation was observed at 30 minutes, after which there was a decline at 1 to 2 hours, followed by a subsequent slight increase at 3 hours (Fig. 9). The change in MAPK phosphorylation was prevented by treatment with specific MAPK inhibitors when given 10 minutes before and after trauma: UO126 (10 μmol/L), an inhibitor of mitogen-activated protein kinase/ERK kinase kinase (the upstream kinase specific for ERK1/2); SB239063 (10 μmol/L), an inhibitor of p38-MAPK; and SP600125 (5 μmol/L), an inhibitor of JNK (data not shown). The concentrations of MAPK inhibitors were selected based on dose-response studies performed for their maximal inhibitory effect on MAPK activation.

Effect of Antioxidants, L-NAME, and CsA on Trauma-Induced MAPK Activation

To determine whether ONS and the mPT contribute to the trauma-induced MAPK phosphorylation, we examined the effect of antioxidants, including catalase (500 U/ml), vitamin E (150 μmol/L), PBN (100 μmol/L), and L-NAME (250 μmol/L), as well as CsA (1 μmol/L) 3 hours after trauma. Doses of these agents were selected based on their optimal concentrations in reducing cell swelling (see succeeding sentences). Treatment of cultures 10 minutes before trauma with vitamin E significantly diminished ERK1/2 and p38-MAPK phosphorylation, but it had no effect on the phosphorylation of JNK. Cultures treated with PBN significantly blocked the phosphorylation of JNK and p38-MAPK, but not phospho-ERK1/2. Catalase, on the other hand, did not block phosphorylation of all 3 MAPKs (Fig. 10). N-Nitro-l-arginine methyl ester significantly diminished the phosphorylation of all 3 MAPKs. Treatment of cultures with the previously described agents 10 minutes after trauma also showed similar effects on MAPKs phosphorylation (data not shown). Cultures treated with CsA (1 μmol/L) 10 minutes before trauma significantly diminished trauma-induced ERK1/2, JNK1/2/3, and p38-MAPK phosphorylation (Fig. 11); FK506 had no effect (data not shown).

FIGURE 8. Cyclosporin A (CsA) (1 μmol/L) significantly blocked astrocyte swelling 3 hours posttrauma. FK506, a calcineurin inhibitor that has no effect on the mitochondrial permeability transition, did not block astrocyte swelling. *, p < 0.05 versus sham control; †, p < 0.05 versus trauma (T).

FIGURE 9. Time course of extracellular signal-regulated kinase (ERK)1/2, c-jun-n-terminal kinase (JNK), and p38–mitogen-activated protein kinase (MAPK) activation after trauma in cultured astrocytes. (A) Western blots reveal a significant increase in phospho-ERK1/2 level when cultures were exposed to 5 atm trauma for 3 hours. Similarly, astrocytes exposed to trauma showed an increase in phospho-JNK1 level. A slight but significant increase in the level of phospho-p38-MAPK was identified after trauma. (B) Quantification of the trauma-induced MAPK activation. Phospho-ERK1/2, JNK, and p38-MAPK levels were normalized against α-tubulin. *, p < 0.05 versus sham control. Con, control.
MAPK Inhibitors Attenuate Trauma-Induced Astrocyte Swelling

Doses of the MAPK inhibitors that prevented MAPK phosphorylation were chosen, and their effect on astrocyte swelling was determined 3 hours after trauma. Treatment of cultures with MAPK inhibitors, including UO126, SB 239063, and SP600125, 10 minutes before trauma significantly decreased astrocyte swelling by 65% to 80% at 3 hours posttrauma (Fig. 12). Cultures treated with the previously described agents 10 minutes after trauma also showed similar effects (data not shown). The Table summarizes the effects of antioxidants, CsA, and MAPK inhibitors on trauma-induced changes.

DISCUSSION

This study demonstrates for the first time that direct mechanical injury to astrocytes in culture results in cell swelling. The swelling occurred in the absence of other injured cells such as neurons which are known to release factors, including Ca\(^{2+}\), potassium and excitatory amino acids, which are believed to contribute to cytotoxic edema/astrocyte swelling in vivo (8). Additionally, trauma caused a significant increase in NO generation, the induction of mPT, and activation of MAPKs. Antioxidants, L-NAME, and the mPT inhibitor CsA and MAPK inhibitors all blocked trauma-induced astrocyte swelling. Antioxidants also significantly diminished the trauma-induced mPT and MAPK activation. These findings suggest that ONS, mPT, and MAPKs are critical factors in the mediation of astrocyte swelling after trauma.

The mechanisms involved in posttraumatic brain edema remain poorly understood. Although both vasogenic (extracellular fluid accumulation due to a breakdown of the blood-brain barrier) and cytotoxic (intracellular fluid accumulation due to failure of cells to regulate their own volume) mechanisms are involved (2), a cytotoxic mechanism (astrocyte swelling) predominates during the early phase of trauma (2–24 hours) (3, 4). Neuroimaging studies further support a cytotoxic mechanism in the early phase of TBI (1). The degree of edema detected in patients on diffusion-weighted magnetic resonance imaging obtained shortly after injury (i.e. cytotoxic edema) correlates highly with a negative clinical outcome (1).

Oxidative/nitrosative stress has been hypothesized to play an important role in the pathogenesis of TBI (30–32), and increased free radicals and associated oxidative stress in the brain have been identified after trauma (33). Increased brain NO levels and protein tyrosine nitration have also been reported in TBI (3, 34). An increase in free radical production

FIGURE 10. (A) Effect of antioxidants on trauma-induced mitogen-activated protein kinase (MAPK) phosphorylation. Cultured astrocytes exposed to trauma (T) significantly increased the phosphorylation of extracellular signal-regulated kinase (ERK)1/2, c-Jun-N-terminal kinase (JNK), and p38-MAPK at 3 hours. Vitamin E did not block JNK activation, although it significantly blocked ERK1/2 and p38-MAPK. N-Tert-butyl-α-phenylnitroxide (PBN) had no effect on ERK1/2 activation, but it significantly blocked JNK and p38-MAPK. N-nitro-L-arginine methyl ester (LN) significantly blocked the activation of all 3 MAPKs. (B) Quantification of the effect of antioxidants on trauma-induced MAPK activation. Catalase had no effect on the activation of MAPKs. Phospho-ERK1/2, JNK, and p38-MAPK levels were normalized against α-tubulin. *, p < 0.05 versus sham control; and †, p < 0.05 versus trauma. CAT, catalase; Vit E, vitamin E.
was shown in cultured astrocytes exposed to trauma (29), and in the present study, we found that NO levels were also elevated.

Free radicals and associated oxidative stress have been shown to induce cell swelling in cultured astrocytes (35–37) and in brain slices (38, 39). Similarly, the involvement of NO in the mediation of astrocyte swelling has also been demonstrated in ammonia toxicity (11, 40), after hypoxic-mimetic swelling (41, 42), and by the use of NO donors (41). The present study demonstrates that antioxidants (except SOD) and L-NAME significantly block astrocyte swelling in our trauma model. Taken with our prior demonstration of trauma-induced ROS production in cultured astrocytes, our present findings indicate an important involvement of ONS in the mediation of astrocyte swelling after trauma.

The results after the use of antioxidants disclosed several incongruities. For example, SOD blocked mPT but not cell swelling, whereas catalase blocked cell swelling but not mPT. Similarly, catalase did not block MAPK activation. It should be emphasized, however, that the precise free radical species generated by trauma in our model are not known. Furthermore, dissimilar free radical species, each possibly appearing at different points and each being at different levels, are likely involved both in vitro and in vivo. Such heterogeneities may have contributed to the differential responses of antioxidants with regard to cell swelling, mPT, and activation of MAPKs, each of which may also have different levels of sensitivity to oxidant damage.

The present study also demonstrates that the mPT induced as early as 10 minutes after trauma was maximal at 3 hours posttrauma. The mPT is characterized by a sudden

**TABLE. Effect of Agents on Trauma-Induced Changes**

| Agents          | Cell Swelling | mPT   | JNK1 | ERK1/2 | p38   |
|-----------------|---------------|-------|------|--------|-------|
| l-NAME          | B             | B     | B    | B      | B     |
| Vitamin E       | B             | B     | NB   | B      | B     |
| Catalase        | B             | NB    | NB   | NB     | NB    |
| SOD             | NB            | B     | ND   | ND     | ND    |
| PBN             | B             | ND    | B    | NB     | B     |
| CsA             | B             | B     | B    | B      | B     |
| MAPK inhibitors | B             | ND    | B    | B      | B     |

B, Blocked; CsA, cyclosporin A; ERK, extracellular signal-regulated kinase; JNK, c-Jun-N-terminal kinase; l-NAME, N-nitro-l-arginine methyl ester; MAPK, mitogen-activated protein kinase; mPT, mitochondrial permeability transition; NB, not blocked; ND, not determined; PBN, N-tert-butyl-o-phenylnitroso; SOD, superoxide dismutase.
increase in the permeability of the inner mitochondrial membrane that is generally triggered by a mitochondrial overload of Ca\(^{2+}\) \((43, 44)\) and frequently occurs as a consequence of oxidative stress \((45, 46)\). Onset of the mPT is associated with a collapse of the \(\Delta \Psi_m\) leading to cellular bioenergetic failure \((for \ reviews, 47–49)\). The involvement of the mPT in TBI has been shown by the CsA-sensitive swelling of brain mitochondria \((50)\). Additionally, CsA reduces the extent of tissue injury after TBI \((50, 51)\).

Maximal induction of the mPT after trauma correlated well with the peak time of astrocyte swelling \((both \ at \ 3 \ hours)\), suggesting that the mPT participates in the cell swelling mechanism. This is further supported by data showing blockade of the trauma-induced astrocyte swelling by CsA. Of relevance is a report noting a reduction of brain edema by CsA in experimental TBI \((52)\). A role for the mPT in astrocyte swelling has also been demonstrated in ammonia neurotoxicity \((53, 54)\) and in manganese toxicity \((55)\). As previously noted, ONS is a major factor in the induction of the mPT. Our findings that antioxidants and \(l\)-NAME blocked the trauma-induced mPT are consistent with the key role of ONS in mPT induction. Catalase, however, did not inhibit the mPT despite the fact that it significantly blocked astrocyte swelling after trauma. Possible explanations for this disparity were previously discussed.

We demonstrate that 10-minute pretreatment of cultures with CsA blocked trauma-induced astrocyte swelling. When administered 10 minutes after trauma, however, CsA did not display an inhibitory effect. The reason for this unexpected finding is not known, but because mPT induction occurred as early as 10 minutes after trauma \(i.e.\) the earliest time the mPT can be measured), such early induction might have prevented any inhibitory effect by CsA on cell swelling.

Precisely how the mPT results in astrocyte swelling after trauma is not known. Although ONS is a major inducer of the mPT \((45, 46)\), it can also be a consequence of the mPT \((56–58)\). As previously noted, several studies have demonstrated that free radicals and associated oxidative stress can cause astrocyte swelling \((35–39)\). These reports are consistent with our earlier study in which we demonstrated that the production of ROS in cultured astrocytes after trauma was significantly blocked by CsA \((29)\). Therefore, ROS production after mPT may represent one mechanism by which mPT leads to astrocyte swelling.

Another mechanism by which the mPT may contribute to cell swelling is by decreasing cellular energy status. It is known that induction of the mPT and associated dissipation of the \(\Delta \Psi_m\) results in decreased oxidative phosphorylation, leading to the loss of ATP \((47)\), which is known to occur in TBI \((59–61)\). In this study, we detected a 27% reduction in ATP in cultured astrocytes 3 hours after trauma, and this ATP loss was completely blocked by CsA treatment. Adenosine triphosphate loss and dissipation of the \(\Delta \Psi_m\) was also noted in a stretch model of \textit{in vitro} trauma \((62)\). Because cell volume regulation requires the effective functioning of various ion transporters, all of which operate at the expense of energy \((63–65)\), the mPT-mediated energy loss may also have contributed to astrocyte swelling.

A major finding in this study was the robust (3-fold) activation of MAPKs, including ERK1/2, JNK1, and p38-MAPK, all of which remained elevated for up to 3 hours after trauma. These data are consistent with activation of MAPKs in rat brain in TBI \((66)\), and the activation was also observed as early as 2 hours after TBI in nonneuronal cerebral cortical cells \((67)\). Activation of ERK1/2 has also been reported in other in vitro models of mechanical trauma \((68, 69)\). Moreover, inhibitors of all 3 MAPKs significantly blocked cell swelling. These findings support a prominent role of MAPKs in astrocyte swelling after trauma. The involvement of MAPKs in cell swelling has also been demonstrated in cultured astrocytes exposed to pathophysiologic levels of ammonia \((11)\).

Like the mPT, MAPKs are also known to be activated by ONS \((11, 70)\). Our present results are consistent with this view in that antioxidants and \(l\)-NAME inhibited MAPK activation \((phosphorylation)\), although antioxidants had variable effects. Thus, vitamin E inhibited ERK and p38-MAPK but not JNK activation, whereas PBN attenuated the activation of JNK and p38-MAPK but not ERK. On the other hand, catalase did not block any of the MAPKs examined, whereas \(l\)-NAME inhibited the activation of all 3 MAPKs. Although our findings demonstrate an important role of ONS in MAPK activation, there seems to be a unique specificity of antioxidants for different MAPKs.

Although this study demonstrates that mechanical trauma result in free radical formation, induction of the mPT, and activation of MAPK, resulting in cell swelling, precisely how trauma leads to these changes is not known. One likely possibility is the activation stretch-activated ion channels \((71)\), in particular, calcium-activated ones \((72)\). Such activation may lead to elevated intracellular Ca\(^{2+}\) levels that are known to occur after trauma \((73)\). Ca\(^{2+}\) elevation can initiate the formation of free radicals as a consequence of activation of nicotinamide adenine dinucleotide phosphate \(\text{oxidized form) oxidase and constitutive NO synthase, as well as by evoking mitochondrial dysfunction (74, 75). The combination of Ca}^{2+} \text{and NO is known to activate MAPKs and to induce the mPT (another source of ROS) (11, 43, 44, 76, 77). Additionally, cytoskeletal disorganization after mechanical trauma can result in increased intracellular Ca}^{2+} \text{(72), ROS production (78), and activation of intracellular signaling kinases, including MAPK (79, 80). It is likely that these early ion and cytoskeletal alterations in traumatized astrocytes may have triggered the changes observed in our study. Although ONS, the mPT, and MAPKs may act independently to produce cell swelling after trauma, there are interactions among these 3 factors. Thus, antioxidants and \(l\)-NAME blocked the induction of the mPT, invoking ONS in the mechanism of trauma-induced mPT. Similarly, antioxidants and \(l\)-NAME attenuated the activation of MAPK, also implicating ONS in the mechanism of MAPK activation after trauma. Additionally, CsA significantly blocked MAPKs presumably by inhibiting ROS production after the mPT, suggesting a role for the mPT in MAPK activation. The latter finding is consistent with an earlier study demonstrating that CsA blocked trauma-induced ROS production \((29)\).
Although the precise pathways by which trauma results in astrocyte swelling remain to be determined, it is apparent that these factors are inextricably involved, and that ONS likely represents a pivotal integrating mechanism. 

In summary, our study indicates that trauma causes cell swelling in cultured astrocytes, and that the swelling seems to be mediated by ONS, the mPT, and activation of mitogen-activated protein kinases. These factors may represent potential targets for the therapy of TBI-associated brain edema.

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