Activation of NF- κ B Mediates Astrocyte Swelling and Brain Edema in Traumatic Brain Injury

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Abstract

Brain edema and associated increased intracranial pressure are major consequences of traumatic brain injury (TBI). While astrocyte swelling (cytotoxic edema) represents a major component of the brain edema in the early phase of TBI, its mechanisms are unclear. One factor known to be activated by trauma is nuclear factor- κ B (NF- κ B). Because this factor has been implicated in the mechanism of cell swelling/brain edema in other neurological conditions, we examined whether NF- κ B might also be involved in the mediation of post-traumatic astrocyte swelling/brain edema. Here we show an increase in NF- κ B activation in cultured astrocytes at 1 and 3 h after trauma (fluid percussion injury, FPI), and that BAY 11–7082, an inhibitor of NF- κ B, significantly blocked the trauma-induced astrocyte swelling. Increased activities of nicotinamide adenine dinucleotide phosphate-oxidase and the Na⁺, K⁺, 2Cl[–] cotransporter were also observed in cultured astrocytes after trauma, and BAY 11–7082 reduced these effects. We also examined the role of NF- κ B in the mechanism of cell swelling by using astrocyte cultures derived from transgenic (Tg) mice with a functional inactivation of astrocytic NF- κ B. Exposure of cultured astrocytes from wild-type mice to *in vitro* trauma (3 h) caused a significant increase in cell swelling. By contrast, traumatized astrocyte cultures derived from NF- κ B Tg mice showed no swelling. We also found increased astrocytic NF- κ B activation and brain water content in rats after FPI, while BAY 11–7082 significantly reduced such effects. Our findings strongly suggest that activation of astrocytic NF- κ B represents a key element in the process by which cytotoxic brain edema occurs after TBI.

Key words: astrocyte swelling; NF- κ B, traumatic brain injury; transgenic mice

Introduction

BRAIN EDEMA is a major neurological complication of traumatic brain injury (TBI),^{1,2} and astrocyte swelling (cytotoxic edema) represents an important component of the brain edema in the early phase after TBI.¹ Mechanisms responsible for astrocyte swelling after trauma, however, are incompletely understood. Oxidative stress (OS) has been hypothesized to play an important role in the pathogenesis of TBI,³ and we previously found that *in vitro* trauma to cultured astrocytes resulted in a significant increase in free radical production, including the formation of nitric oxide.^{4,5} Oxidative stress is also known to phosphorylate (activate) mitogen-activated protein kinases (MAPKs), including p38-MAPK, c-Jun N-terminal kinase (JNK), and the extracellular signal-regulated kinase (ERK). MAPK phosphorylation has also been described after TBI *in vivo*³ and *in vitro*.⁴

Reactive oxygen species and MAPKs activation (phosphorylation) are also well known to stimulate various transcription

factors—in particular, nuclear factor- κ B (NF- κ B).^{6,7} Activation of NF- κ B was recently shown to cause swelling of cultured astrocytes after exposure to ammonia⁸ and to also be involved in the brain edema associated with acute liver failure.⁹ Activation of NF- κ B has likewise been documented in the brain after TBI.^{10–14} The precise role of this transcription factor in the development of astrocyte swelling/brain edema following TBI is not known.

The present study examined whether activation of NF- κ B occurs in astrocytes after trauma, *in vitro* and *in vivo*, and whether such activation contributes to the astrocyte swelling/brain edema after TBI. We found that activation of NF- κ B is increased after trauma to cultured astrocytes, and that blockade of such activation with a pharmacological inhibitor, or by using cultured astrocytes from transgenic (Tg) mice with a functional inactivation of astrocytic NF- κ B, attenuated such swelling. In addition, increased NF- κ B activation was detected in astrocytes after TBI in rats, and BAY 11–7082, an inhibitor of NF- κ B, significantly reduced the brain water content after TBI. These findings suggest that NF- κ B plays a

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central role in the development of astrocyte swelling/brain edema associated with TBI.

Methods

Astrocyte cultures

Astrocyte cultures were prepared from brains of 1-day-old rat pups, as well as from transgenic (Tg) mice pups, as described previously.^{9,15} Tg mice expressing I- κ B α -dn under control of the glial fibrillary acidic protein (GFAP) promoter (GFAP-I- κ B α -dn) were generated at the Transgenic Animal Core Facility of the University of Miami.¹⁶ Briefly, cerebral cortices were freed of meninges, minced, dissociated by trituration and vortexing, passed through sterile nylon sieves, placed in Dulbecco modified Eagle medium containing penicillin, streptomycin, and fetal bovine serum, and incubated at 37°C in a humidified chamber 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-cAMP (Sigma) so as to enhance cell differentiation.¹⁷

Fluid percussion injury (FPI) model of in vitro trauma

The fluid percussion instrument used was initially described by Sullivan and associates in 1976¹⁸ and subsequently modified for cell culture by Shepard and colleagues in 1991¹⁹ and Panickar and coworkers in 2002.⁵ Briefly, astrocyte cultures grown in individual 35-mm culture dishes were placed in the injury chamber that 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 (atm) of pressure were administered twice for a 25-msec 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 and colleagues in 1975,²⁰ as modified by Bender and Norenberg in 1998.²¹ Briefly, 1 mM 3-*O*-methylglucose and 0.5 μ Ci/ml ³H-labeled 3-*O*-methylglucose were added to the cultures immediately after trauma. At the end of the incubation period, culture medium was aspirated, and an aliquot was saved for radioactivity determination. Cells were rapidly washed and harvested into 0.5 mL of 1N sodium hydroxide. Radioactivity in the cell extracts and medium were determined, and an aliquot of the cell extract was used for protein estimation (bicinchoninic acid method [BCA] method). Values were normalized to protein level, and cell volume was expressed as μ L/mg of protein.

Preparation of nuclear extracts and immunoblots of NF- κ B

Nuclear extracts were prepared as previously described.⁸ In brief, astrocytes were harvested in phosphate buffered saline and centrifuged. The cell pellet was resuspended in 10 mM HEPES buffer with a complete protease inhibitor cocktail (Roche, Mannheim, Germany). The pellet was then incubated on ice for 15 min, after which 15 μ L of 10% NP-40 was added, and the sample vortexed thoroughly for 40 sec. An aliquot of the homogenate was used for protein measurement. An equal amount of protein was then centrifuged at 3000 rpm for 3 min at 4°C. The resulting cell pellet was resuspended in 20 mM HEPES buffer and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant (containing the nuclear extract) was analyzed for NF- κ B content by Western blots as described previously.^{8,22} An antibody against NF- κ B-p65 (H-286)

(cat# sc-7151; 1:750 Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was used to detect NF- κ B. Lamin A/C (a nuclear protein) antibody was purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated antimouse and antirabbit secondary antibodies (Vector Laboratories, Burlingame, CA) were used at 1:1000 dilutions.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity

Cells exposed to different experimental conditions were washed three times with Hanks balanced salt solution, and NOX activity in the cell extract was measured by a chemiluminescence assay as described previously.²³ Results were expressed as luminescence intensity/min/mg protein. Protein content in homogenates was measured by the BCA method (Pierce, Rockford, IL).

Na⁺, K⁺, 2Cl⁻ cotransporter (NKCC) activity

NKCC activity was measured as the bumetanide-sensitive K⁺ influx, using ⁸⁶Rb as a tracer for K⁺ by a modification of the method of Sun and Murali in 1999.²⁴ Briefly, 3 h after trauma, cultures were incubated with or without 50 mM bumetanide for 15 min at 37°C in an isotonic HEPES-minimal essential medium. Cultures were then exposed to 1 μ Ci/mL ⁸⁶Rb for 3 min and rinsed with ice-cold 0.1 M MgCl₂. Cells were then extracted in 1% sodium dodecyl sulfate, and the radioactivity was analyzed by liquid scintillation. ⁸⁶Rb influx rate was calculated by subtracting the influx (with bumetanide) from total influx (without bumetanide), as described previously,^{24–26} and expressed as nmol of ⁸⁶Rb/mg of protein/min.

Fluid percussion brain injury in vivo

Before injury (24 h), rats were anesthetized (70% nitrous oxide, 1–3% isoflurane, and 30% oxygen) and surgically prepared for a parasagittal FPI as described previously.²⁷ Briefly, a craniotomy (4.8 mm) was performed 3.8 mm posterior to the bregma and 2.5 mm lateral to the midline. A plastic injury tube was placed over the exposed dura and affixed to the skull. The scalp was then sutured closed, and the animals were allowed to recover before returning to their home cages. After fasting overnight, animals were reanesthetized (70% nitrous oxide, 1–3% isoflurane, and 30% oxygen) and intubated. The femoral artery was cannulated to ensure blood gases, pH, glucose, and mean arterial blood pressure (MABP) measurements were consistent among animals. Rectal and temporalis muscle thermometers measured core and brain temperatures using self-adjusting feedback warming lamps. All variables were maintained within physiological ranges from 15 min before TBI and for up to 45 min post-injury.

A moderate (1.8–2.2 atm) fluid percussion-induced TBI was produced over the right parietal cortex.²⁸ Sham animals underwent identical procedures except for the insult. The treatment group received intravenous injection of BAY 11-7082 (5 mg/kg body weight), an inhibitor of NF- κ B, or its solvent (0.5% methyl cellulose in distilled water) 5–10 min after trauma.

Immunohistochemistry of cortical nuclear NF- κ B

Three hours after trauma or sham procedure, rats were transcardially perfused with heparinized saline for 1 min, followed by fixation in 4% paraformaldehyde for 15 min. After decapitation, heads were left in the same fixative for an additional 24 h. Brains were cryoprotected (12–24 h) in 30% sucrose, and 20 micron-thick cortical sections obtained with a cryostat. Sections were blocked with 10% goat serum and incubated overnight at 4°C with antibodies specific to the p65 subunit of NF- κ B (1:200 dilution; Santa Cruz Biotech, Inc., Santa Cruz, CA) and to GFAP (astrocyte marker, 1:150 dilution Abcam, Cambridge, MA) as described previously.²⁹

After incubation with primary antibodies, sections were washed with tris-buffered saline (TBS) containing 1% Tween-20 (TBS-T) and incubated with fluorescent horseradish peroxidase-conjugated secondary antibodies (1:500; AlexaFluor-Rhodamine for GFAP and Alexa Fluor-FITC for NF- κ B) for 2 h. Sections were then covered with commercial mounting media (Vector Labs.) containing 4',6-diamidino-2-phenylindole (DAPI) (nuclear stain). Immunofluorescent images were acquired with a Zeiss LSM510/UV Axiovert 200 M confocal microscope with a plan apochromat 40 $\times$ objective lens and a 2 $\times$ zoom, resulting in images of 125 $\times$ 125 mm in area and 1.0 mm optical slice thickness (1.0 Airy units for Alexa Fluor 546 or 568 emission channel).

Random collection of images from sections of control and rats with TBI was achieved by systematically capturing each image in a blinded manner by moving the microscope stage approximately 5 mm in all four different directions. At least 15 fluorescent images were captured per rat, and the images were merged to localize astrocytic nuclear NF- κ B expression. Pixel volumes of these images (DAPI plus green) were quantified using the Volocity 3D Image analysis software (Volocity 6.0 High Performance 3D Cellular Imaging Software Suite; PerkinElmer) as described previously.^{30,31} Values were expressed as percent change of intensity units of blue/green ratios (DAPI/NF- κ B).

Brain water measurement

Brain water content was determined by the wet/dry weight method as described previously.³² Briefly, both sham and rats with TBI were decapitated 3 h post-trauma, and the brains were immediately removed. Approximately 10 mg cortical sections were weighed fresh, desiccated at 110°C for 48 h, and then reweighed. The percent brain water content in each section was determined by differences in wet-dry weight.

All procedures followed guidelines established by the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by our Institutional Animal Care and Use Committee.

Statistical analysis

All experiments were performed and repeated 5–7 times using cells derived from different batches of astrocyte cultures. The number of individual culture plates used in each experimental group was six for astrocyte swelling and four for Western blot studies. Seven animals from each group were used for *in vivo* studies. The data of all experiments were subjected to analysis of variance followed by Tukey *post hoc* comparisons. A value of $p < 0.05$ was considered significant. Error bars represent mean $\pm$ standard error of the mean.

Results

1. Trauma activates NF- κ B in cultured astrocytes

Cultured astrocytes were exposed to trauma (5 atm), and changes in nuclear NF- κ B expression (activation) were determined at different time points after trauma (30 min, 1, 2, and 3 h) by Western blots of the nuclear fraction. A significant increase in NF- κ B activation was first observed at 1 h after trauma, which remained elevated for up to 3 h (71.4, 51.8, and 58.6%, at 1, 2, and 3 h after trauma, respectively; $p < 0.05$ vs. control) (Fig. 1).

2. Inhibition of NF- κ B activation results in reduced cell swelling in cultured astrocytes after FPI

We previously demonstrated that astrocyte cultures exposed to trauma caused cell swelling.⁴ We now examined the effect of NF- κ B inhibition on the trauma-induced astrocyte swelling.

FIG. 1. Representative Western blots of nuclear factor- κ B (NF- κ B) expression in nuclear extracts of cultured astrocytes after trauma. Increased nuclear NF- κ B was identified in cultured astrocytes at 1, 2, and 3 h after trauma (5 atm pressure). (A) Western blots of nuclear NF- κ B; (B) Quantification of Western blots. NF- κ B content was normalized against lamin A/C, a nuclear protein. Analysis of variance, $n = 6$. * $p < 0.05$ vs. control.

Traumatized astrocytes were post-treated (5–7 min) with different concentrations of NF- κ B inhibitors: BAY 11-7082 (1, 3, 5, and 10 μ M) and SN50 (1, 2, and 3.5 microM), and cell volume was measured 3 h later. Trauma caused astrocyte swelling by 56% ($p < 0.05$, compared with sham), and treatment of cells with BAY 11-7082 (5 μ M) and SN50 (2 microM) significantly diminished the trauma-induced astrocyte swelling (58% and 63%, respectively, $p < 0.05$) (Fig. 2). The concentrations of NF- κ B inhibitors were selected based on dose-response studies (BAY 11-7082 [1, 3, 5 and 10 μ M] and SN50 [1, 2 and 3.5 μ M]) performed for their maximal inhibitory effect on NF- κ B activation. We found that the activation of NF- κ B after trauma was maximally inhibited at these concentrations (data not shown).

3. Cultured astrocytes from Tg mice with a functional inactivation of astrocytic NF- κ B showed no cell swelling after trauma

To more definitively examine the role of NF- κ B on astrocyte swelling after trauma, we used astrocyte cultures from Tg mice with a functional inactivation of astrocytic NF- κ B¹⁶ and examined the effect of trauma on cell volume in these cells. Astrocyte cultures from Tg mice exhibited no swelling when exposed to 5 atm trauma. By contrast, trauma significantly increased cell swelling (32% over control) in cultured astrocytes from wild type (WT) mice (Fig. 3). The cell volume of control Tg cells was not statistically significantly different from that found in control cells of WT astrocytes.

4. NF- κ B inhibition diminishes the trauma-induced NOX activation

One important consequence of NF- κ B activation is the stimulation of NOX,^{33,34} and such activation of NOX is known to mediate astrocyte swelling after hyperammonemia.^{35,36} We therefore

FIG. 2. Effect of nuclear factor- κ B (NF- κ B) inhibitors on trauma-induced cell swelling in cultured astrocytes exposed to 5 atm trauma. Cultures were treated (5–7 min) with different concentrations of NF- κ B inhibitors: BAY (BAY 11-7082) (1, 3, 5, and 10 μ M) and SN50 (1, 2, and 3.5 μ M), and cell volume was measured 3 h later. Trauma increased astrocyte swelling by 56% ($p < 0.05$, compared with sham). Treatment with optimal doses of BAY (5 μ M) or SN50 (2 μ M) significantly diminished the trauma-induced astrocyte swelling (58% and 63%, respectively). Analysis of variance, $n = 5$. * $p < 0.05$ vs. control; † $p < 0.05$ vs. trauma. Cont, control.

examined whether trauma-induced NF- κ B activation contributes to NOX activation. Cultures exposed to trauma caused an increase in NOX activity, and BAY 11-7082 (5 μ M) significantly diminished this increase by 47% ($p < 0.05$) (Fig. 4).

5. NF- κ B inhibition diminishes trauma-induced NKCC activity in cultured astrocytes

We previously showed that astrocytes exposed to trauma resulted in a significant increase in the NKCC activity, and that its inhibition with bumetanide diminished the cell swelling in cultured astrocytes after trauma.²⁶ Because activation of NF- κ B has been strongly implicated in the stimulation of various ion channels/exchangers in other conditions,^{37–40} we examined whether NF- κ B activation likewise stimulates NKCC activity after trauma to cultured astrocytes, and whether such activation contributes to the astrocyte swelling.

Astrocytes were exposed to trauma (5 atm), and changes in NKCC activity were determined after 3 h by measuring the bumetanide-sensitive K^+ influx, using ^{86}Rb as a tracer for K^+ , with and without BAY 11-7082. Cultures exposed to 5 atm pressure showed a statis-

FIG. 3. Comparison between astrocyte cultures from wild-type (WT) mice vs. transgenic (Tg) mice in which nuclear factor- κ B (NF- κ B) was inactivated. Trauma significantly increased cell swelling (32% over control) in cultured astrocytes derived from WT mice. In contrast, astrocyte cultures from Tg mice exhibited no swelling when exposed to 5 atm trauma. Cell volume in control group from WT mice astrocytes were not significantly different from Tg controls. Analysis of variance, $n = 5$. * $p < 0.05$ vs. WT control; † $p < 0.05$ vs. Tg control and WT trauma. C, control; T, trauma.

tically significant increase in NKCC activity at 3 h, while treatment with BAY 11-7082 (5 μ M) significantly diminished the trauma-induced NKCC activity (48.6%, $p < 0.05$) (Fig. 5A).

In addition to the effect of pharmacological suppression of NF- κ B on NKCC activity, we also examined whether silencing NF- κ B expression in astrocytes similarly diminishes the trauma-induced activation of NKCC. Astrocytes were therefore transfected with an siRNA targeted to rat NF- κ B p65 smart pool of three target-specific 20–25 nt siRNAs designed to knock down the expression of p65 mRNA. At the end of transfection, total RNA was isolated, and mRNA and protein levels of NF- κ B p65 were determined by reverse transcription polymerase chain reaction and Western blots, respectively. We found a significant reduction (78.5%) in mRNA levels of NF- κ B p65, and a 51.4% decrease in NF- κ B p65 protein expression after transfection with 100 nM siRNA for 48 h (Fig. 5B,C). We also found that silencing NF- κ B p65 (using 100 nmoles of siRNA) reduced the trauma-induced NKCC activity (by 56.8%) compared with sham control (Fig. 5D). A concentration of 100 nmoles of NF- κ B p65 siRNA was found to be optimal, because lower concentrations (25 and 50 nmoles) did not efficiently silence the expression of NF- κ B p65, whereas higher concentrations (0.50 and 1 mM) resulted in cytotoxicity.

Similar findings were observed in cultured astrocytes from Tg mice with a functional inactivation of astrocytic NF- κ B; i.e., a significant increase in NKCC activity was detected in traumatized astrocytes derived from WT mice, whereas astrocyte cultures from

FIG. 4. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity in cultured astrocytes after trauma. Astrocyte cultures exposed to 5 atm trauma for 3 h significantly increased NOX activity, while post-treatment (5–7 min) of cells with BAY (BAY 11-7082) significantly reduced such effect. Analysis of variance, $n=4$. * $p < 0.05$ vs. control; † $p < 0.05$ vs. trauma. Cont, control.

Tg mice exhibited a reduction in NKCC activity after trauma (data not shown). These findings indicate that NF- κ B contributes to the trauma-induced NKCC activation.

6. Nuclear NF- κ B expression in cerebral cortex from rats after TBI

To examine whether alterations in nuclear NF- κ B expression also occur *in vivo* after TBI, FPI to the right parietal lobe was performed in rats and 3 h later the extent of nuclear NF- κ B expression was examined in cortical sections. To identify changes in nuclear NF- κ B in astrocytes, sections were co-immunostained with GFAP and DAPI. Semi-quantitative analysis of astrocytic nuclear NF- κ B expression using the Volocity 3D Image program showed an increase in nuclear NF- κ B (96%) after TBI compared with control rats (Fig. 6). We also measured NF- κ B activation at 9 days after trauma and found a significant increase (71.8%) in nuclear NF- κ B activation (figure not shown).

7. NF- κ B inhibition attenuates trauma-induced brain edema in rats

After FPI (1.9 atm), brain water content (edema) was determined 5 mm peripheral to the site of injury (i.e., 5 mm surrounding the epicenter). We measured brain edema at different time points after trauma (1–24 h), and found the peak increase in edema at 3–6 h after trauma (3.14 and 3.07%, at 3 and 6 h after trauma, respectively; 81.23 ± 0.2 in sham controls vs. 84.37 ± 0.2 in traumatized rats at 3 h after trauma). Treatment of rats (5–10 min after trauma) with BAY 11-7082 (5 mg/kg body weight), showed a 75.62% reduction in brain edema at 3 h after trauma (Fig. 7). Treatment of animals with 5 mg/kg body weight BAY 11-7082, in the absence of trauma, had no significant effect on brain water content (data not shown). A

slight increase (0.41%) in brain water content was detected in sham animals compared with normal control (i.e., normal control animals without surgery) (81.23% in sham vs. 80.82% in normal control). This increase in brain water content in sham animals, however, was not statistically significant different from normal control.

The concentration of the NF- κ B inhibitor (BAY 11-7082, 5 mg/kg body weight) used in this study was selected based on our previous findings showing that BAY 11-7082 significantly reduced the brain edema in acute liver failure.⁴¹ In addition, we also used lower and higher concentrations of BAY 11-7082 (1 and 10 mg/kg body weight) to examine the effect of NF- κ B inhibition on the brain edema. While we found a significant inhibition in the brain edema after treatment of rats with 1 and 10 mg/kg body weight of BAY 11-7082, the effect of 5 mg/kg was greater than that observed with 1 and 10 mg/kg body weight (Fig. 7). Doses above 10 mg/kg (25 mg/kg) had no effect on the brain edema after trauma.

In addition, we examined animal survival for up to 3 weeks after treatment with the NF- κ B inhibitor BAY 11-7082 (5 mg/kg), with and without trauma. Traumatized animals that were treated with the NF- κ B inhibitor showed a similar survival to that of sham control at 21 days after trauma. All physiological parameter in sham control and TBI rats were within normal range throughout the experiments (Table 1).

Discussion

Our study demonstrates that astrocyte cultures exposed to trauma caused an increase in NF- κ B nuclear translocation (activation), and that pharmacologic blockade of NF- κ B activation or genetic deletion of astrocytic NF- κ B reduced the extent of trauma-induced astrocyte swelling. Further, trauma-induced increases in NOX and NKCC activities were diminished by NF- κ B inhibition. In addition, the increase in brain water content in rats after TBI was reduced by BAY 11-7082. These studies strongly suggest that activation of NF- κ B represents an important factor in the mechanism of cytotoxic brain edema in the early phase after TBI.

NF- κ B is known to be activated in response to oxidative stress, as well as by inflammatory mediators.⁴² Activation of NF- κ B results in the induction of several inflammatory/oxidative stress responsive genes,⁴² which play a major role in cell injury in different neurological conditions.⁴³ Its activation has also been documented in brains after TBI.^{10–13} Whether NF- κ B is involved in the development of astrocyte swelling/brain edema after TBI is not known. The present study found that FPI to cultured astrocytes resulted in the activation of NF- κ B and that pharmacological inhibition of such activation significantly reduced the trauma-induced astrocyte swelling. In addition, the involvement of NF- κ B in cell swelling was shown in astrocyte cultures from Tg mice with a functional inactivation of astrocytic NF- κ B. These cultures showed an absence of swelling when exposed to trauma, in contrast to cultured astrocytes derived from WT mice.

The mechanism(s) by which a direct mechanical injury to cultured astrocytes results in the activation of NF- κ B and subsequent cell swelling is unclear. We recently found that astrocyte cultures exposed to trauma (mechanical injury) increased the generation of free radicals,⁵ activation of the mitochondrial permeability transition (mPT), and MAPKs,⁴ and that blockade of these events significantly diminished the astrocyte swelling.⁴ Because activation of NF- κ B was previously shown to occur in response to enhanced free-radical production, induction of the mPT or activation of MAPKs,^{7,44} it is possible that one or more of these events after trauma may have contributed to the activation of NF- κ B.

FIG. 5. Effect of nuclear factor- κ B (NF- κ B) inhibition on Na^+ , K^+ , 2Cl^- cotransporter (NKCC) activity after trauma. Cultured astrocytes were exposed to 5 atm trauma, and the bumetanide-sensitive NKCC activity was measured with and without BAY 11-7082 3h later. **(A)** Trauma significantly increased NKCC activity at 3 h, and such effect was diminished by treatment of astrocytes with BAY-11 7082. We also examined whether silencing NF- κ B gene expression in cultured astrocytes similarly diminishes the trauma-induced activation of NKCC. **(B)** Astrocytes transfected with an siRNA targeted to NF- κ B p65 showed a significant reduction in mRNA levels of NF- κ B p65 **(C)** as well as a decrease in NF- κ B p65 protein expression. **(D)** Silencing of NF- κ B significantly reduced the trauma-induced NKCC activity. Analysis of variance, $n = 7$ * $p < 0.05$ vs. control. † $p < 0.05$ vs. trauma. Cont, control; Scr, scrambled siRNA.

The means by which activation of NF- κ B contributes to cell swelling in traumatized astrocyte cultures is unclear. One potential factor known to be activated by NF- κ B is the induction of OS.⁶ Activation of NOX by NF- κ B represents an important factor in the induction of OS, as NOX is one of the most important sources of superoxide formation.^{33,34} Activation of NOX has been shown to stimulate cell swelling in cultured astrocytes after ammonia treatment.³⁶ Consistent with these observations, we found increased NOX activity after trauma in cultured astrocytes and that BAY 11-7082, an inhibitor of NF- κ B, significantly diminished such activation. In addition, apocyanin, an inhibitor of NOX, partially reduced the trauma-induced cell swelling in cultured astrocytes (data not shown). These findings suggest that the NF- κ B-induced cell swelling after trauma could partly be mediated by increased NOX activity.

Another factor that may be involved in the trauma-induced astrocyte swelling by NF- κ B is the activation of the ion transporter NKCC1. We previously showed that astrocytes exposed to trauma resulted in a significant increase in NKCC activity, and that its

inhibition with bumetanide diminished the cell swelling in cultured astrocytes after trauma.²⁶ As activation of NF- κ B has been strongly implicated in the stimulation of various ion channels/exchangers in other conditions,^{37–40} we examined whether NF- κ B activation likewise stimulates NKCC activity after trauma to cultured astrocytes, and whether such activation contributes to astrocyte swelling. A significant increase in NKCC activity was observed after trauma, and BAY-11 7082 blocked such activity, suggesting that the NF- κ B-mediated cell swelling after trauma is, in part, a consequence of increased NKCC activity.

It is not clear how activation of NF- κ B alters NKCC activity after trauma. We previously demonstrated that activation of NF- κ B increases the production of free radicals⁸ resulting in the oxidation of NKCC1 in ammonia-treated astrocyte cultures.²⁵ Such oxidation led to the stimulation of NKCC1 phosphorylation (activation) and the subsequent increase in cell volume in cultured astrocytes.^{25,26} It is therefore possible that similar events may occur after trauma.

FIG. 6. Activation of nuclear factor- κ B (NF- κ B) in astrocytes in cerebral cortex of rats after traumatic brain injury (3 h). (A, F), 4',6-diamidino-2-phenylindole (blue, nuclei); (B, G) NF- κ B (green); (C, H) glial fibrillary acidic protein (red, astrocytes). Sham (control) brain: (D) merged image of A and B illustrates the basal level of NF- κ B in nuclei (arrows) and (E) merged image of A–C exemplifies the normal levels of NF- κ B in astrocytic nuclei. Trauma brain: (I) merged image of F and G demonstrates increased nuclear NF- κ B (arrows) compared with control brain. (J) Merged image of F–H illustrates increased levels of NF- κ B in astrocytic nuclei. Scale bar = 20 μ m (J).

FIG. 7. Effect of BAY 11-7082 on the brain edema after traumatic brain injury (TBI). A significant increase in brain water content was detected at 3 h after TBI (3.14%, compared with sham control). Treatment of rats with BAY 11-7082 showed a reduction in brain edema. Analysis of variance, $n=5$. * $p<0.05$ vs. control. † $p<0.05$ vs. trauma. C, control; T, trauma.

Conclusion

Astrocyte cultures exposed to trauma induces NF- κ B nuclear translocation (NF- κ B activation) as well as increases in NOX and NKCC activities. Blockade of NF- κ B by genetic manipulation or with pharmacological inhibition significantly reduces the extent of NOX and NKCC activities as well as the cell swelling in cultured astrocytes after trauma. In addition, activation of NF- κ B and increased brain water content were detected in rats after TBI, and treatment of rats with the NF- κ B inhibitor BAY 11-7082 caused a

TABLE 1. PHYSIOLOGIC PARAMETERS*

	TBI drug	TBI control	Sham drug
Pre-trauma			
pH	7.43 $\pm$ 0.01	7.37 $\pm$ 0.04	7.42 $\pm$ 0.01
pCO ₂ (mm Hg)	42.10 $\pm$ 0.49	43.23 $\pm$ 0.92	43.67 $\pm$ 0.12
pO ₂ (mm Hg)	125.17 $\pm$ 5.52	126.53 $\pm$ 9.02	118.00 $\pm$ 4.36
MABP (mm Hg)	105.33 $\pm$ 10.14	104.67 $\pm$ 12.97	122.33 $\pm$ 8.82
Brain temperature (°C)	36.30 $\pm$ 0.08	36.38 $\pm$ 0.11	36.33 $\pm$ 0.12
Rectal temperature (°C)	36.53 $\pm$ 0.26	36.75 $\pm$ 0.14	36.61 $\pm$ 0.24
Post-trauma			
pH	7.45 $\pm$ 0.01	7.42 $\pm$ 0.02	7.42 $\pm$ 0.02
pCO ₂ (mm Hg)	40.28 $\pm$ 1.05	39.62 $\pm$ 0.91	43.60 $\pm$ 0.47
pO ₂ (mm Hg)	100.58 $\pm$ 4.16	115.83 $\pm$ 6.75	108.67 $\pm$ 7.69
MABP (mm Hg)	106.50 $\pm$ 4.82	108.50 $\pm$ 9.09	131.00 $\pm$ 8.33
Brain temperature (°C)	36.53 $\pm$ 0.15	36.33 $\pm$ 0.09	36.60 $\pm$ 0.12
Rectal temperature (°C)	36.94 $\pm$ 0.07	36.49 $\pm$ 0.17	36.67 $\pm$ 0.10

*Mean $\pm$ standard error of the mean.

All physiological variables were within normal range throughout the experiments.

MABP, mean arterial blood pressure.

reduction in the brain edema. These findings strongly suggest that the activation of NF- κ B in astrocytes is an integral component of the development of cytotoxic brain edema after TBI, and that therapeutic approaches targeting astrocytic NF- κ B may represent a useful strategy for the treatment of the brain edema in the early phase of TBI.

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Author Disclosure Statement

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