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Synergistic Inhibition of ERK1/2 and JNK, Not p38, Phosphorylation Ameliorates Neuronal Damages After Traumatic Brain Injury

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Abstract

Mitogen-activated protein (MAP) kinases are serine/threonine protein kinases that play a critical role in signal transduction and are activated by phosphorylation in response to a variety of pathophysiology stimuli. While MAP kinase signaling has a significant role in the pathophysiology of several neurodegenerative diseases, the precise function of activation of MAP kinase in traumatic brain injury (TBI) is unknown. Therefore, it is important to study the role of MAP kinase signaling in TBI-associated neurological ailments. In this study, using an in vitro stretch injury model in rat embryo neuronal cultures and the in vivo fluid percussion injury (FPI) model in rats, we explored the role of MAP kinase signaling in the mechanisms of cell death in TBI. Our study demonstrated that the stretch injury in vitro and FPI in vivo upregulated the phosphorylation of MAP kinase proteins ERK1/2 and JNK, but not p38. Using ERK1/2 inhibitor U0126, JNK inhibitor SP600125, and p38 inhibitor SB203580, we validated the role of MAP kinase proteins in the activation of NF-kB and caspase-3. By immunofluorescence and western blotting, further, we demonstrated the role of ERK1/2 and JNK phosphorylation in neurodegeneration by analyzing cell death proteins annexin V and Poly-ADP-Ribose-Polymerase p85. Interestingly, combined use of ERK1/2 and JNK inhibitors further attenuated the cell death in stretch-injured neurons. In conclusion, this study could establish the significance of MAP kinase signaling in the pathophysiology of TBI and may have significant implications for developing therapeutic strategies using ERK1/2 and JNK inhibitors for TBI-associated neurological complications.

Keywords Traumatic brain injury · Fluid percussion injury · Mitogen-activated protein kinase · Neurodegeneration

Introduction

Traumatic brain injury (TBI) is one of the leading causes of death and disability worldwide [1]. In the USA, the rate of TBI occurrence is every 15 s causing 1.7 million new head injury victims per year. TBI events are responsible for 50,000 deaths and 80,000 individuals with permanent disabilities with an estimated cost of more than US$77 billion on average per year [2]. Until now, there is no known effective pharmacological intervention to mitigate the adverse neurological outcomes of TBI, because the underlying biochemical mechanisms are not fully elucidated [1, 3].

Previous studies in both in vitro and in vivo models of CNS traumatic injury have demonstrated that mitogen-activated protein kinase (MAPK) signaling pathway cascade is activated in response to mechanical trauma [4, 5]. MAPKs are serine/threonine protein kinases that play a critical role in signal transduction that is activated by phosphorylation in response to a variety of injury stimuli [6]. The cascade of events associated with MAPK signaling pathways involves extracellular signal-regulated protein kinase (ERK), p38, and c-Jun NH (2)-terminal kinase (JNK) pathways [6]. Initially, it was considered that ERK1 and 2 (isoforms of ERK) are activated by receptor tyrosine kinases or G protein-coupled receptors and act as a promoter in the regulation of cellular proliferation and differentiation, thus playing a significant role in neuronal survival and memory [7]. In contrast to ERK, JNKs and p38 kinases are activated by cytokines and stress signals and are implicated in growth arrest, apoptosis, and inflammatory responses. However, an intriguing debate has emerged regarding the functional roles of MAPKs following injury stimulus. Recent studies have demonstrated that ERK activation in response to growth factors [8], oxidative stress [9], and

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intracellular calcium influx [10] can trigger neuronal death signals [11]. One line of evidence suggests that TBI upregulates ERK and p38 MAPK pathways with no detectable change in JNK pathway and that pharmacologic inhibition of the ERK [11, 12] or p38 signaling pathway [13] significantly improves cell survival in both in vitro and in vivo models of trauma. Another line of study suggests that activation of ERK and JNK but not p38 cascades may be closely involved in signal transduction following TBI [13, 14] and inhibition of JNK signaling pathway could be a potential therapeutic approach in reducing the progression of pathological features in TBI [15]. Although previous in vitro and in vivo studies show activation of MAPKs following TBI, it remains elusive how the mechanism of differential activation MAPK signaling pathway plays a key role in the causation of TBI.

In recent studies, we have demonstrated that oxidative stress incurred during TBI leads to activation of downstream signaling pathways such as activation of MMPs, disruption of the blood-brain barrier, neuroinflammation, and neurodegeneration [16–22]. Oxidative stress is also associated with activation of TGF-β1 cascade leading to neuroinflammation and neurodegeneration via phosphorylation of Smad proteins [21]. Previous studies suggest crosstalk mechanisms between MAP kinase pathways and Smad signaling downstream of TGF-β activation [23]. These results demonstrate oxidative stress as a key mediator of neuroinflammation and neurodegeneration that can lead to the activation of the MAPK pathways [24] playing a significant role in the pathophysiology of TBI. Therefore, MAP kinase signaling pathway would be a potential therapeutic target in ameliorating the pathophysiology associated with TBI. Herein, the purpose of this study is to elucidate the mechanisms by which activation of MAP kinase pathway triggers neurodegeneration using an in vitro neuronal stretch injury model and validate the pathway in an animal model of fluid percussion injury (FPI). In the present study, we propose the idea that TBI or neuronal injury activates the phosphorylation of ERK and JNK that promotes the exacerbation of injury-induced neuroinflammation and neurodegeneration. We used U0126 (ERK kinase (MEK1/2) inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 MAP kinase inhibitor) to block the activation of ERK, JNK, and p38 in vitro, respectively, to promote recovery from brain injury complications. We also analyzed the synergistic effect of U0126 and SP600125 in reducing injury-associated neurodegeneration in vitro. Hence, targeting ERK and JNK signaling cascade with pharmacological inhibitors presents new therapeutic possibilities for the treatment of TBI-related neurological diseases.

Material and Methods

Primary Cortical Neuronal Cultures

All experimental procedures involving animals were approved by the Seton Hall University Institutional Animal Care and Use Committee (IACUC). All procedures were performed in accordance with the strict guidelines of the 8th edition of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The cerebral cortical neuronal culture was prepared as previously described (Muneer et al. 2016, 2017) from E17 Sprague-Dawley rat embryos. Briefly, cerebral cortices from rat brain were rinsed with Ca^{2+} and Mg^{2+} free HBSS followed by digestion with 0.25% trypsin containing EDTA (0.2 g/L) (Sigma-Aldrich, Cat. No. T4049) and DNase I (1.5 mg/mL) at 37 °C for 30 min, neutralized with 10% fetal bovine serum, and further dissociated by trituration. The unwanted large pieces of tissue were removed by filtrating dissociated cells using 100 and 40 μm pore-sized cell strainers (EMD Millipore, Billerica, MA). Neurons were cultured on a poly-D-lysine (0.1 mg/mL) coated elastic silicone membrane mounted on Bioflex culture plates (Flexcell International Corp, Burlington, NC) at a density of 300,000 cells/well and incubated at 37 °C with 5% CO_2. Cells were cultured in NeuroBasal media containing 0.4 mM L-glutamine, 2% B-27, and 1% penicillin-streptomycin. The cells were fed by changing the medium every 2 days, and neurons were grown to confluence before use. The purity of neurons was assessed by immunofluorescence with the neuronal marker, NeuN, to confirm 100% enrichment of neurons.

In Vitro Neuronal Stretch Injury

Following day 10 of neuronal culture, the cells were exposed to stretch injury (biaxial) with a pressure of 2.0 psi using an in vitro cell injury device, "cell injury controller II" (Custom design and fabrication Inc., Sandstom, VA) [16, 21, 25, 26]. Control neurons were also cultured in the stretch injury culture plates but not subjected to stretch injury. For experiments with pharmacological treatments, neuronal culture was treated with ERK1/2 inhibitor, U0126 (10 μM); JNK inhibitor, SP600125 (30 μM); p38 inhibitor, SB203580 (30 μM); and NOX inhibitor, apocynin (100 μM), 30 min prior to the stretch injury. The dose 10 μM U0126 was selected from three best doses 5, 10, and 15 μM by analyzing its inhibitory effect on the phosphorylation of ERK1/2. Similarly, the dose of SP600125 (30 μM) and SB203580 (30 μM) was selected from 10, 20, and 30 μM. Twenty-four-hour post-stretch injury, cell culture supernatant was collected for cytokine analysis and cells were fixed for immunostaining and proteins were extracted for western blotting. For temporal resolution of phosphorylated ERK1/2 and JNK, cell culture supernatant and protein lysates were collected and prepared at regular time intervals (0, 6, 12, 24, and 48 h) after stretch injury.

In Vivo FPI

FPI and sham injury procedures were performed in 9-week-old male Sprague-Dawley rats (250–300 g; Taconic

Biosciences) based on our standard protocols previously described [16, 21]. Briefly, under ketamine/xylazine (mixture of 80 mg/kg ketamine and 10 mg/kg xylazine) anesthesia, a 3-mm craniotomy was performed at 3.0 mm posterior from and 3.5 mm lateral from the bregma in a stereotaxic device to create a circular window exposing the intact dura of the brain. A hollow Luer-Lok syringe hub was then secured over the craniotomy window by Cranioplastic cement (AM Systems, Carlsborg, WA) to ensure effective fluid transmission and support. During the surgery process, body temperature (T_b) was continuously monitored and maintained within normal ranges (36.5–37.5 °C) by a feedback temperature controller pad (model TC-1000; CWE Ardmore, PA). Twenty-four hours later, the animal was anesthetized with 5% isoflurane until the foot-pinch reflex stopped. The animal was then attached to a digitally controlled fluid percussion injury system-FP302 (AmScien Instruments, Richmond, VA) via the syringe hub, and the fluid pressure was applied at 7–8 and 15–18 psi (eight animals each) with a pressure rise time of 8 ms. The animal was monitored for duration of the apnea, loss of consciousness (hind-paw withdrawal), and latency to occurrence of the self-righting reflex immediately after sham or FPI injury to access acute injury severity [27]. On resumption of breathing, the syringe hub was removed and the wound sutured and closed. Similarly, control animal underwent the same procedures as the FPI injury rat except that the fluid pulse was not given. We used eight rats each in injured and control groups for this study. 6, 12, 24, and 48 h following TBI injury, the animals were perfused transcardially with ice-cold 1× phosphate-buffered saline (PBS; pH 7.2–7.4) followed by 4% paraformaldehyde in PBS. The brains were removed, post-fixed in 4% paraformaldehyde for 4 h at 4 °C, and then immersed in 30% sucrose until the brains sank to the bottom of the sucrose solution. Brain tissues were dissected out, embedded in an OCT (optimal cutting temperature) compound, and kept frozen until analysis.

Western Blotting

For westernblotting, cells (three wells of similar condition) or tissue samples (~ 50 mg) were collected and sonicated in homogenizing buffer (Thermo Scientific, Rockford, IL) containing a mixture of protease inhibitor (Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged at 14,000 rcf for 10 min at 4 °C to remove unbroken cells and debris. The resulting supernatant collected was then quantified for total protein concentration using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL). Immunoblots were performed by resolving the protein (20 μg) in 4–15% gradient SDS-PAGE gel (Biorad, Hercules, CA), and the blots were transferred onto a nitrocellulose membrane and blocked with superblock (Thermo Scientific, Rockford, IL) for 1 h. The membranes were incubated with primary antibodies against ERK1/2, p-ERK1/2, JNK, p-JNK, NF-kB (1:1000, Cell Signaling, Danvers, MA), Cleaved caspase-3 (1:1000; R and D, location), annexin V (1:1000; Thermofisher, location), Poly-ADP-Ribose-Polymerase p85 (PARP p85) (1:1000; Abcam, Cambridge, MA, USA), and β-actin (1:1000; Thermofisher, St. Louis, MO) for overnight at 4 °C. After washing three times at 5-min intervals, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:5000; Fisher Scientific, Rockford, IL). The membrane was rinsed three times with TBS-tween for 5 min at RT. Protein bands were detected using chemiluminescence western blot detection reagents (Abnova, Walnut, CA) and scanned with an imager, Syngene gel documentation system (Frederick, MD). The optical density was quantified as arbitrary densitometry intensity units using the ImageJ software package (NIH). Protein of interest was normalized and quantified using β-Actin as a loading control.

Immunofluorescence and Microscopy

Immunofluorescence staining was performed in both cultured neurons and 10-μm thickness cryostat-sectioned coronal brain tissue sections as previously described [21, 28–30]. Briefly, the cultured neurons and the brain sections were washed with 1× PBS and fixed in 4% paraformaldehyde for 20 min at 25 °C. The sections were then immersed in 1× PBS (pH 7.4) for 5 min and then blocked in 3% normal goat serum containing 0.1% Triton X-100 (5% heat inactivated BSA, PBS at pH 7.4) for 1 h at room temperature. The cell culture membrane or tissue sections were then incubated for 24 h at 4 °C with the primary antibodies against PARP p85 (1:250; Abcam, Cambridge, MA, USA), annexin V (1:250; Thermo Scientific, Rockford, IL, USA), and NeuN (1:250; Thermofisher, St. Louis, MO). All antibodies were used at the concentration of 3.0 μg/mL. Cells or tissue sections were then washed in 1× PBS at room temperature for 15 min and incubated with secondary antibodies (Alexa Fluor 488 or 594 conjugated with anti-mouse or anti-rabbit immunoglobulin G (IgG); 1:500 dilution or 4 μg/mL) for 1 h and mounted with 5–10 μL immunomount containing DAPI (Invitrogen) on a slide.

As previously described [16, 21], for semiquantitative analysis, the protein of interest was captured using the Eclipse TE200 fluorescent microscope (Nikon, Melville, NY) and NIS Elements software (Nikon, Melville, NY) in three channels, DAPI (cell nuclei), Alexafluor-488 (green color emission), and Alexafluor-594 (red color emission). For imaging, the area of image capture was randomized and was performed by a researcher that was blinded to the experimental conditions. Analysis was performed on at least four tissue samples for immunofluorescent staining and captured six images from a single sample (slide). Just below the injury or the peri-lesion

area, approximately 1.5 mm^2 area was covered for the analysis. For consistency during image capture, same parameters of camera and software including brightness of the excitation light, the detector sensitivity (gain), or the camera exposure time across the samples or tissue sections for in vitro and in vivo studies were used. The intensity of immunostaining was analyzed by ImageJ (NIH) software. To determine the percentage of positive cells for a particular protein of interest, the threshold of the images was maintained by keeping lower threshold level to 80 and upper threshold level to 200. To correct for uneven illumination in fluorescence images, uniform dark background for all images was kept.

Cell Death Analysis

Apoptotic cell death in cultured neurons and brain tissue sections were determined by western blotting using anti-cleaved PARP p85 (1 mg/mL; Promega, Madison, WI) and annexin V (1 mg/mL, Thermo Scientific, Rockford, IL, USA) primary antibodies. Anti-cleaved PARP p85 identifies the cleaved 85 kDa PARP fragment of 116 kDa PARP, which is an early marker for apoptosis and is mediated by a caspase-3 signaling pathway.

Statistical Analysis

Data processing and statistical analyses were performed using Microsoft Office Excel 2010 and GraphPad Prism 6. Data were analyzed with one-way or two-way ANOVA with repeated measures. Significant main effects or interactions were subjected to Bonferroni's correction. Statistical significance was accepted at the 95% confidence level. Data are expressed as mean ± SEM, and $p < 0.05$ was considered for statistical significance.

Results

TBI Activates ERK1/2 and JNK Signaling Cascades In Vivo and In Vitro

To investigate the role of MAP kinase signaling pathway in the mechanisms of neurodegeneration following brain injury, initially, we studied the activation of MAP kinase signaling pathway in mild (7 psi) and moderate (15 psi) fluid-percussion model of traumatic brain injury (TBI) in rat. Brain slices from FPI injured and control group animals were used to analyze the expression level of three major MAPK pathways: ERK, JNK, and p38. In the parietal region of the cortex below the site of injury, western blot data shows that a significant high level of activation of ERK and JNK phosphorylation in both 7 psi ($p < 0.01$) and 15 psi ($p < 0.001$) FPI groups 24 h after injury compared to uninjured control animals (Fig. 1a, b). However, there were no detectable changes in the phosphorylation of p38 following TBI (Fig. 1c). In addition, there was no noticeable change in the levels of the total amounts of ERK, JNK, and p38 proteins after injury compared with control groups. Next, we analyzed the expression level of p-ERK and p-JNK in 6–48 h period at 6, 12, 24, and 48 h time intervals 7 psi FPI rat brain tissues. The expression level of p-ERK and p-JNK significantly increased at 12, 24, and 48 h compared to 6 h following FPI (Fig. 1d, e). However, upregulation of p-ERK and p-JNK persisted at 24 h tapered at 48 h. These results confirmed that following mild and moderate FPI, ERK and JNK signaling pathways are activated that might in part contribute to the pathophysiology of TBI.

Next, the FPI-induced activation of MAP kinase pathway (ERK and JNK, p38) was validated in stretch-injured (2.0 psi pressure) cortical neuronal culture by western blotting. A significant upregulation of p-ERK1/2 and p-JNK proteins level was found at 24 h post-injury compared to uninjured cells ($p < 0.01$) (Fig. 2a, b). Further, to study the MAP kinase signaling pathway more precisely, injury-induced activation of phosphorylation of MAP kinase pathway was verified in stretch-injured cortical neuronal culture by blocking the MAP kinase pathway with the treatment of U0126 (ERK kinase inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 MAP kinase inhibitor) independently for 24 h. Treatment of cortical neurons with U0126 reduced the expression level of p-ERK1/2 significantly ($p < 0.001$) (Fig. 2a). Similarly, treatment of a broad-spectrum JNK inhibitor, SP600125 caused a significant decrease in the phosphorylation of JNK (Fig. 2b). However, no significant changes in the expression of p-p38 were observed in injured tissue lysates (Fig. 2c). Interestingly, we found significant reduction in the level of p-p38 with the treatment of p38 MAP kinase inhibitor, SB203580 (Fig. 2c). In addition, no significant changes in the total amounts of ERK, JNK, and p38 proteins were observed after injury compared with uninjured cells. The expression level of p-ERK and p-JNK also analyzed in 0–48 h period at 0, 6, 12, 24, and 48 h time intervals after stretch injury conducted at 2 psi pressure. Interestingly, expression level of p-ERK increased significantly at 12, 24, and 48 h compared 0 h (just after injury) and p-JNK increased significantly at 6, 12, 24, and 48 h compared 0 h following 2 psi stretch injury (Fig. 2d, e). However, upregulation of p-ERK and p-JNK persisted at 24 h tapered at 48 h. These data confirm that stretch-injured neurons activate the phosphorylation of ERK1/2 and JNK proteins time dependently.

In our previous works, we reported the induction of reactive oxygen species (ROS) and oxidative stress-inducing enzymes NOX1 and iNOS and their representative markers such as 4HNE and 3NT in stretch-injured neurons and FPI brain tissue samples [16, 17, 21]. However, we have not shown the link between oxidative stress and phosphorylation of MAP kinase proteins. In this study, since oxidative stress is linked

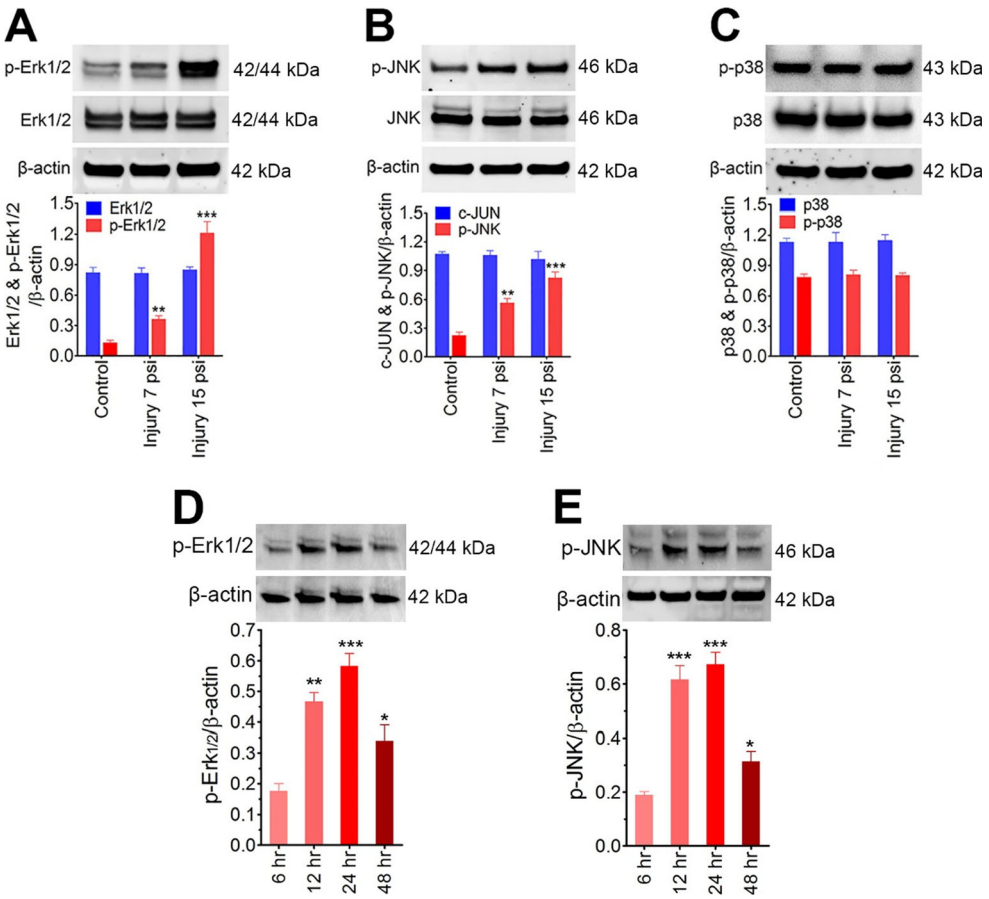

Fig. 1 TBI activates ERK1/2 and JNK signaling cascades in vivo. **a**–**c** Western blot analysis of MAP kinase proteins ERK and p-ERK (**a**), JNK and p-JNK (**b**), and p38 and p-p38 (**c**) and β-actin in rat cortex tissue lysates from uninjured control and FPI samples (7 and 15 psi) 24 h after injury. **d**–**e** Western blot analysis of p-ERK1/2 (**d**) and p-JNK (**e**) and their representative ß-actin expression at different time periods (6, 12, 24, 48 h) in rat cortical tissue lysates after 7 psi F PI. Bar graphs show the results expressed as ratio of MAP kinase proteins and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group in **a**–**e**. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control in **a**-**c**, and $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus 6 h in **d** and **e**

to the activation of the MAP kinase-signaling pathway [24, 31], we analyzed the role of oxidative stress in the phosphorylation of ERK1/2, JNK, and p38 in TBI. The neuronal cultures were pretreated with NADPH oxidase (NOX) inhibitor apocynin (100 μM) and the expression level of p-ERK1/2, p-JNK, and p-p38 were analyzed. In stretch-injured cells, western blot analysis shows that treatment with apocynin significantly attenuated the expression level of p-ERK and p-JNK 24 h after stretch injury compared to untreated injured cells (2 psi) ($p < 0.01$) (Fig. 2a, b). Interestingly, even though there was no significant level of phosphorylated p38 (p-p38) that was observed in injured cells, in apocynin treatment, the level of p-p38 significantly reduced compared to untreated uninjured (control) and injured cells (Fig. 2c). This data suggests that oxidative stress has a significant role in the activation of MAP kinase cascades ERK1/2, JNK and p38.

Activation of MAP Kinase Signaling Cascades Upregulates NF-kB Expression

Activation of NF-kB transcription factor plays a pivotal role in the regulation of several genes involved in response to inflammation and mediates cell death [32]. Since TBI involves a massive amount of inflammatory stimulus, herein, we investigated whether MAP kinases can participate in the regulation of NF-kB transcriptional activity. Western blotting data shows that the expression of NF-kB was significantly increased in both 7 psi ($p < 0.05$) and 15 psi ($p < 0.001$) FPI samples 24 h after injury when compared with uninjured controls (Fig. 3a). To further evaluate, if inhibition of ERK and JNK modulates the activation of NF-kB transcription factor, cells were treated with U0126 (ERK kinase inhibitor) and SP600125 (JNK inhibitor) independently for 24 h. Western blot result shows that ERK inhibition significantly decreased the expression of NF-kB in stretch-injured neurons compared to untreated injured cells ($p < 0.001$) (Fig. 3b). Similarly, a significant decrease in the expression of NF-kB was observed in SP600125-treated cells ($p < 0.001$) (Fig. 3b).

Since oxidative stress modulates MAP kinase signaling pathway, we next investigated NF-kB activation in apocynin-treated cells. We observed that treatment of apocynin significantly decreased the expression level of NF-kB 24-h post-stretch injury ($p < 0.001$) (Fig. 3b). Next, we analyzed the synergistic effect of both ERK and JNK inhibitors in the regulation of NF-kB by treating the cells with U0126 and SP600125 together. Western blotting data shows a further significant decrease in the expression level of NF-kB in stretch-injured cells treated with both U0126 and SP600125

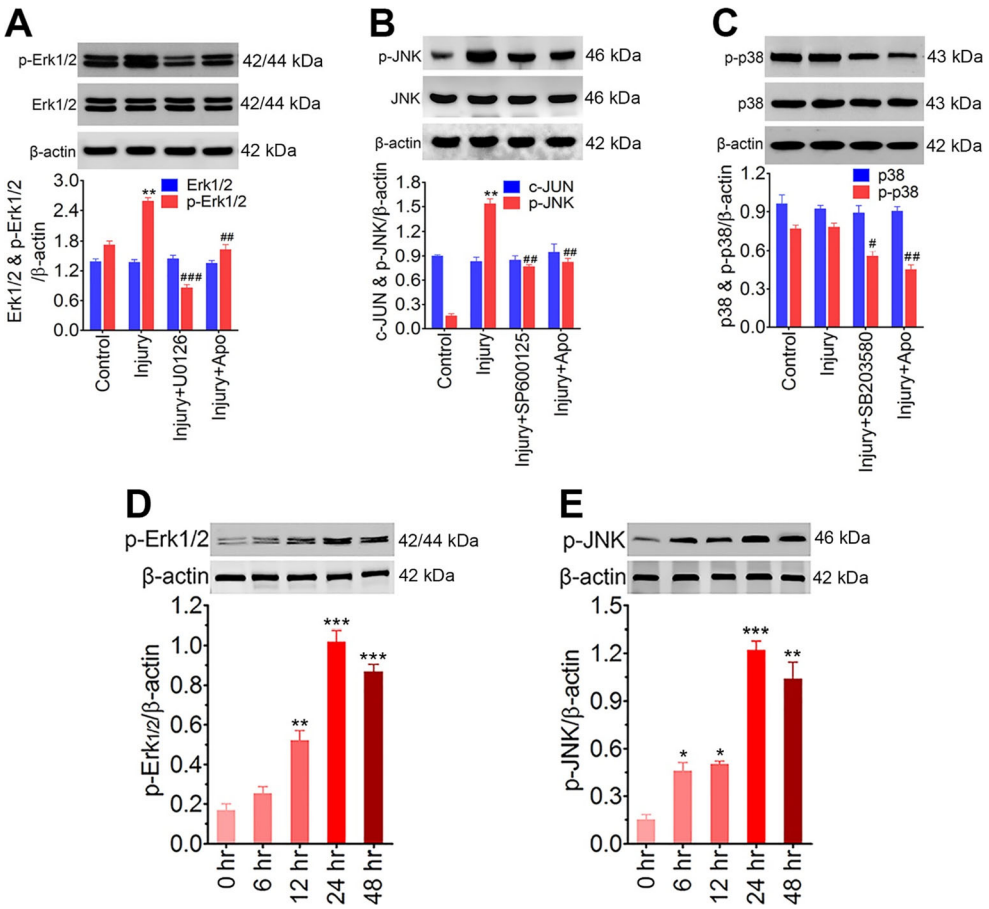

Fig. 2 Stretch injury upregulates the phosphorylation of MAP kinase proteins ERK1/2 and JNK; inhibitors modulate it. **a–c** Western blot analysis of Erk1/2 and p-Erk1/2 (**a**), JNK and p-JNK (**b**), p38 and p-p38 (**c**) from uninjured control, injury (2 psi), injury + MAP kinase inhibitors (ERK1/2 inhibitor U0126 in A, JNK inhibitor SP600125 in B, and p38 kinase inhibitor SB2035802 in **c**), and injury + apocynin after 24 h of treatment. **d–e** Western blot analysis of p-ERK1/2 (d) and p-JNK (**e**) and their representative ß-actin expression at different time points (0, 6, 12, 24, 48 h) in rat cortical neuronal lysates after 2 psi stretch injury. Bar diagram represents the results expressed as ratio of MAP kinase proteins and β-actin bands. All values are represented as mean ± SEM and n = 6/group in **a–e**. $^{**}p < 0.01$ versus control and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.01$versus Injury in a-c; $^{*}p < 0.05$, $^{**}\boldsymbol{p} < 0.01$ and $^{***}p < 0.001$ versus 0 h in **d** and **e** . Apo = apocynin

when compared with either of the individual inhibitor-treated cells ($p < 0.05$) (Fig. 3b). Taken together, these data demonstrate that both ERK and JNK pathways are involved in the regulation/ activation of NF-kB pathway.

MAP Kinase Signaling Pathway Activates Caspase-3

To validate whether activation of MAP kinase cascades leads to apoptotic cell death in neurons, we analyzed the expression of a cleaved form of caspase-3, an apoptotic inducing enzyme [33, 34], in stretch-injured neuronal culture subjected to 2 psi injury. Western blot data demonstrates a significant increase ($p < 0.01$) in the expression of cleaved caspase-3 that was significantly reduced ($p < 0.05$) with pretreatment of U0126 or SP600125 in stretch-injured cells 24 h after injury (Fig. 4a). The combined treatment of U0126 and SP600125 further decreased the expression of cleaved caspase-3 in injured cells ($p < 0.05$) (Fig. 4a).

Since ERK and JNK signaling cascade is dependent on the activation of oxidative stress (Fig. 3b), we next investigated the activation of cleaved caspase-3 in apocynin-treated stretch-injured cells. In western blotting, apocynin significantly reduced the expression of cleaved caspase 3 when compared to untreated injured cells (Fig. 4a). To validate the in vitro results, animals were subjected to FPI at 7 and 15 psi pressures and analyzed for cleaved caspase-3 expression. Western blot data demonstrates a significant upregulation of cleaved caspase-3 protein from the tissue lysates of rat cerebral cortex of 7 and 15 psi FPI animals compared to uninjured animals (Fig. 4b).

MAP Kinase Signaling Has a Significant Role in TBI-Induced Apoptotic Cell Death

To establish the role of MAP kinase in neuronal stretch injury associated cell death, we assessed the extent of cell death using immunofluorescent staining and western blotting of cleaved Poly-ADP-Ribose-Polymerase (PARP) (p85 fragment) and annexin V. By immunofluorescence, it was apparent that stretch injury increased the expression of PARP p85 ($p < 0.001$) that was blocked with pretreatments of U0126 or SP600125 ($p < 0.001$) (Fig. 5a, b). Figure 5b shows the intensity of PARP p85 expression by immunofluorescent staining. Western blot analysis validated the changes in PARP p85 protein expression. A significant upregulation of PARP p85 protein level was found at 24 h post-injury compared to uninjured cells ($p < 0.001$) (Fig. 5c). In U0126-, SP600125-, or apocynin-treated injured cells, the protein level of PARP p85

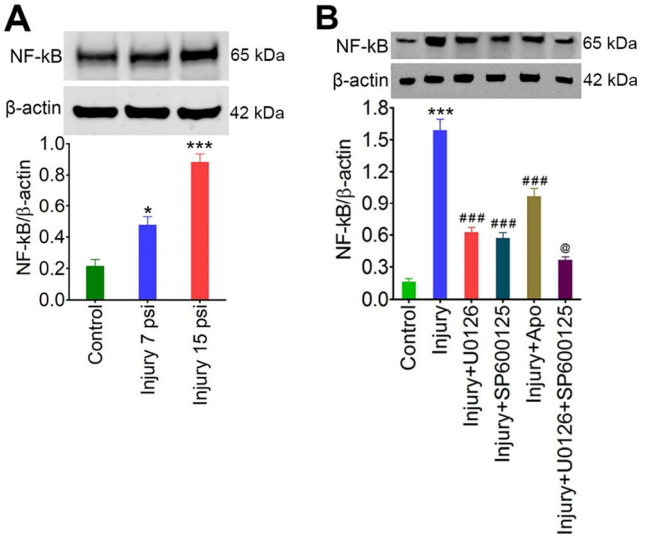

Fig. 3 ERK and JNK regulate the expression of NF-kB. **a** Western blot analysis of NF-kB and β-actin proteins in rat cortical tissue lysates from uninjured control and FPI samples (7 psi and 15 psi) 24 h after injury. Bar graphs show the results expressed as ratio of NF-kB and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control. **b** Western blot analysis of NF-kB from uninjured control, injury (2 psi), injury + U0126, injury + SP600125, injury + apocynin and injury + U0126 + SP600125 after 24 h of treatment. Bar diagram represents the results expressed as ratio of NF-kB and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group. $^{***}p < 0.001$ versus control, $^{###}p < 0.001$ versus injury, and $^{@}p < 0.05$ versus injury + U0126 or injury + SP600125. Apo = apocynin

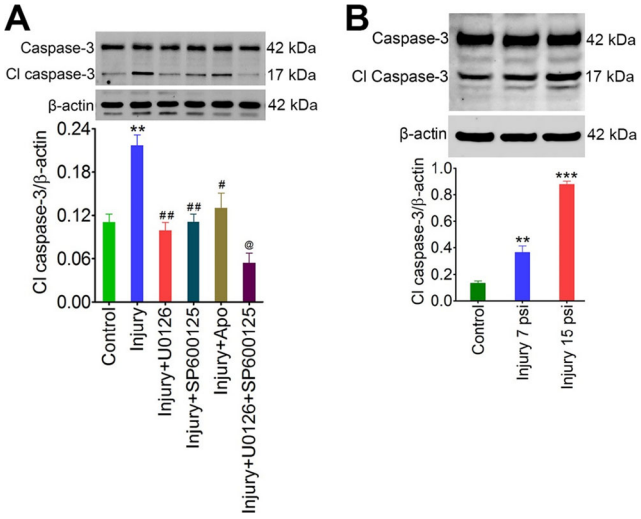

Fig. 4 ERK and JNK activate caspase-3. **a** Western blot analysis of cleaved caspase-3 from uninjured control, injury (2 psi), injury + U0126, injury + SP600125, injury + apocynin and injury + U0126 + SP600125 after 24 h of treatment. Bar diagram represents the results expressed as ratio of cleaved caspase-3 and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group. $^{**}p < 0.01$ versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus injury, and $^{@}p < 0.05$ versus Injury+U0126 or Injury+SP600125. **b** Western blot analysis of cleaved caspase-3 and β-actin proteins in rat cortical tissue lysates from uninjured control and FPI samples (7 and 15 psi) 24 h after injury. Bar graphs show the results expressed as ratio of cleaved caspase-3 and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control. Apo = apocynin, Cl caspase-3 = cleaved caspase-3

was significantly reduced ($p < 0.001$) when compared with untreated injured cells 24 h after injury (Fig. 5c). A similar trend of results was observed in the immunofluorescence of annexin V, and the expression was significantly increased ($p < 0.001$) when compared to uninjured control cells, whereas U0126, SP600125 or apocynin reduced its expression (data not shown). In western blotting, stretch injury increased the expression of annexin V ($p < 0.001$) that was blocked with pretreatments of U0126, SP600125 or apocynin ($p < 0.001$) (Fig. 5d). Moreover, stretch-injured neurons pretreated with the combination of the U0126 and SP600125 show additive neuroprotection when compared to pretreatment with either U0126 or SP600125. This indicates that ERK and JNK signaling cascade acts synergistically in the pathology of TBI, and the effect is dependent on oxidative stress.

We further validated ERK and JNK cascade-mediated cell apoptosis in rat brain cortex tissue sections from control and FPI (7 and 15 psi) animals for PARP and annexin V expressions. In the parietal region of the cortex below the site of injury, in immunofluorescence, the expression level of annexin V was significantly very high in both 7 and 15 psi FPI samples when compared with uninjured samples (Fig. 6a, b). Co-localizing annexin V with NeuN staining indicated that annexin V expression was primarily in neurons. Figure 6b shows the intensity of annexin V expression. A similar trend of results was observed in the expression of PARP p85, and it was significantly increased in both 7 and 15 psi ($p < 0.001$) when compared to uninjured control samples ($p < 0.001$) (Fig. 6c, d). Similarly, annexin V ($p < 0.01$) and the 85 kDa of PARP fragment ($p < 0.01$) western blot bands were highly expressed in FPI animals compared to uninjured controls (Fig. 6e, f). Taken together, these results clearly indicate the role of MAP kinase proteins in neurodegeneration in the form of cell apoptosis in TBI.

Discussion

MAPK signaling pathway has been implicated in the regulation of neuroinflammation and neurodegeneration in a variety of neurological diseases [35–40]. However, the mechanism and role of MAP kinase signaling cascade after TBI are poorly understood. In the present study, we demonstrate that ERK1/2 and JNK signaling cascades are activated following TBI injury both in vivo and in vitro, whereas no significant changes in activation were evident in p38 signaling cascade. Oxidative stress propagates the activation of ERK1/2 and JNK signaling pathway following TBI. Moreover, we also demonstrated that upregulation of phosphorylated ERK1/2 and JNK mediates the upregulation of NF-kB transcription factor leading to the

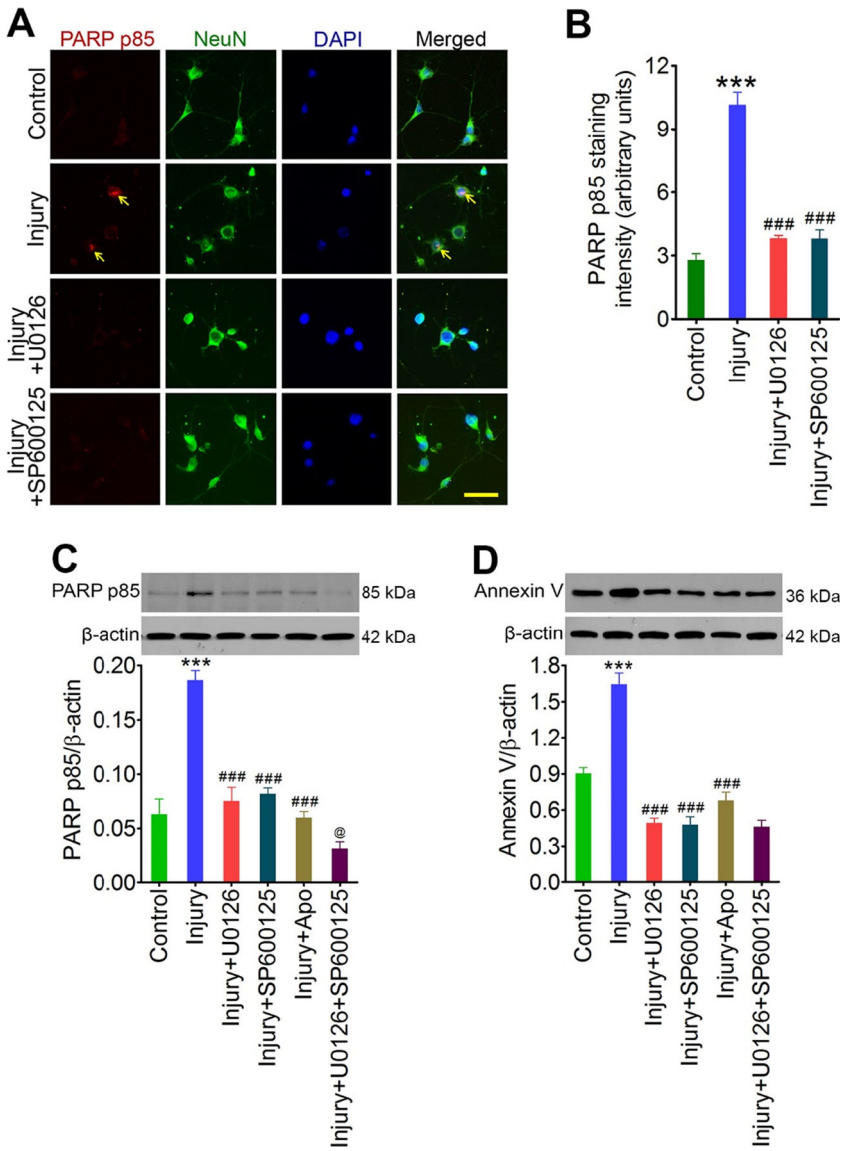

Fig. 5 U0126 and SP600125 mitigate apoptosis in stretch-injured neurons. **a** Immunofluorescent staining of PARP p85 (red) merged with NeuN (green) and DAPI (blue) in uninjured control, injury, injury + U0126, and injury + SP600125 after 24 h of treatment. Yellow arrows indicate high-level expression of PARP p85 in injured neurons. Scale bar = 20 μm. **b** Quantification of PARP p85 staining in cultured neurons from rat brain cortex analyzed using ImageJ software. Values are mean ± SEM and $n = 4$/group. $^{***}p < 0.001$ versus control and $^{\#\#\#}p < 0.001$ versus injury. **c**, **d** Western blot analysis of Parp-p85 (**c**) and annexin-V (**d**) and β-actin proteins in uninjured control, injury (2 psi), injury + U0126, injury + SP600125, injury + apocynin, and injury + U0126 + SP600125 after 24 h of treatment. Bar diagram represents the results expressed as ratio of PARP p85 (**c**) or annexin V (**d**) and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group. $^{***}p < 0.001$ versus control, $^{\#\#\#}p < 0.001$ versus injury, and $^{@}p < 0.05$ versus Injury + U0126 or Injury + SP600125 in (D). Apo = apocynin

activation of caspase-3 causing apoptotic cell death and neurodegeneration.

MAP kinases are a conserved evolutionary family of enzymes that play an essential role in the regulation of gene expression that controls fundamental cellular processes such as cell differentiation, cell proliferation, and cell death [41, 42]. However, the functional role of MAP kinase signaling cascade is more complicated and involves heterogeneous nature of mechanisms in the pathophysiology of various neurological diseases [35–40]. In TBI, recent studies have anticipated that inhibitors of MAP kinase signaling cascade will have efficacy in the treatment of TBI patients [15]. However, there are several conflicting outcomes. Our study provides compelling evidence that following FPI injury modeled in vivo or stretch injury modeled in vitro, activation of ERK and JNK signaling cascades predominates without any significant activation in the p38 signaling cascade. Our study involves the use of U0126, SP600125, and SB203580 as a pharmacological inhibitor to investigate the pathological role of MAP kinase pathways in TBI. Inhibition of ERK pathway with U0126 and JNK pathway with SP600125 was protective against in vitro stretch injury, whereas the p38 inhibitor, SB203580, exhibits no significant effect. Our results show that no significant upregulation of total amounts of ERK, JNK, and p38 was following TBI. These findings suggest that a transient phosphorylation of ERK and JNK after TBI does not attribute to alteration in the expression of ERK or JNK [14].

Oxidative stress is crucial in the progression of neurodegeneration. Activation of NOX acts as one of the critical factors in mediating ROS and contributes significantly to the neuropathology of TBI [16, 17, 20, 21, 43–46]. The effects of ROS generation on MAP kinase pathways have been reported to be involved in pathogenesis of Alzheimer's disease,

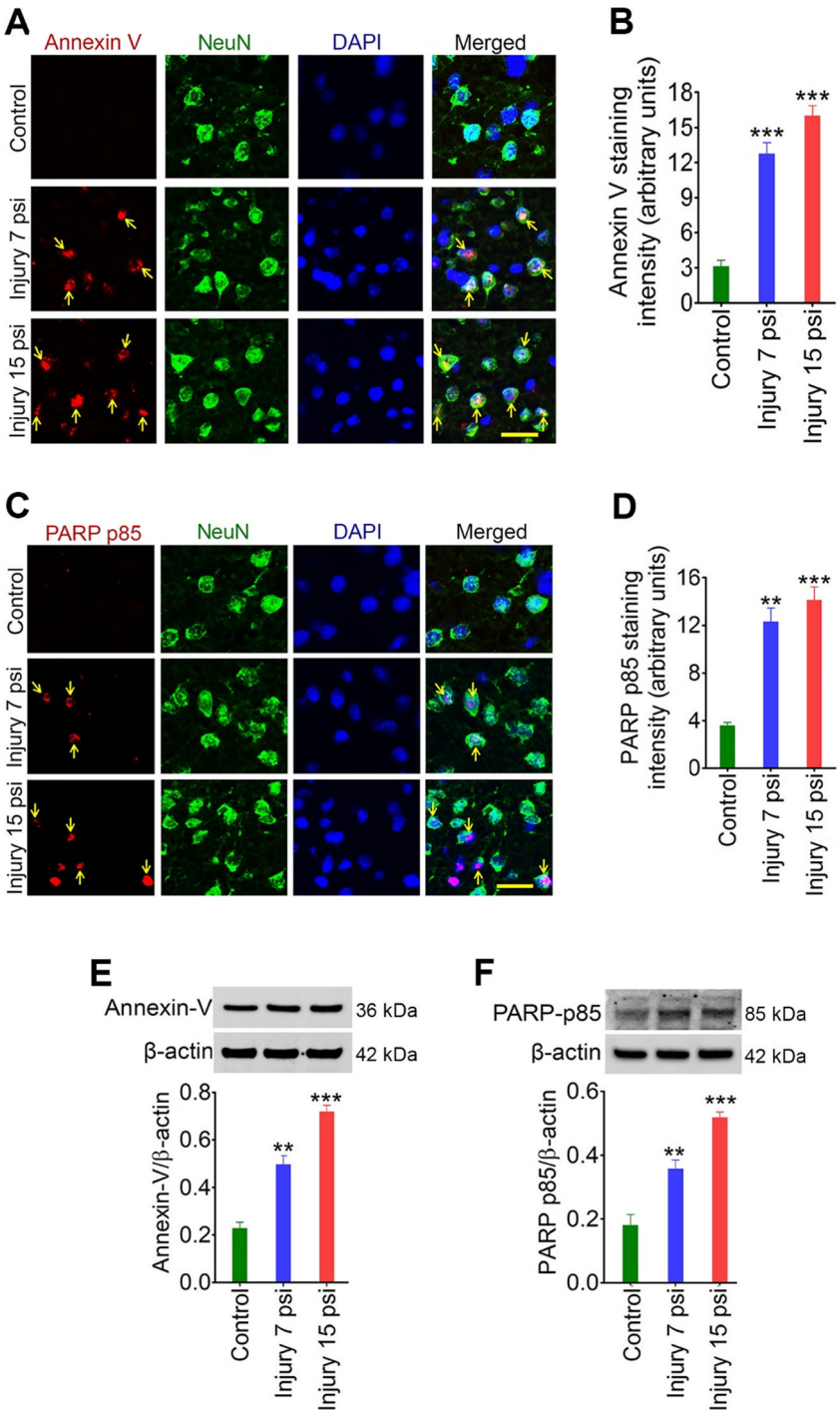

Fig. 6 ERK and JNK induce apoptosis in the animal model of TBI. **a**, **c** Immunofluorescent staining of annexin V (red) (**a**) and PARP p85 (red) (**c**) merged with NeuN (green) and DAPI (blue) in uninjured control, injury 7 psi and injury 15 psi 24 h after FPI. Yellow arrows indicate high-level expression of annexin V or PARP p85 in injured neurons. Scale bar = 20 μm in both **a** and **c**. **b**, **d** Quantification of annexin V or PARP p85 staining in cortical brain tissue sections analyzed using ImageJ software. Values are mean ± SEM and $n = 4$/group. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. **e**, **f** Western blot analysis of annexin V (**e**) and PARP p85 (**f**) and β-actin proteins in rat cortical tissue lysates from uninjured control and FPI samples (7 and 15 psi) 24 h after injury. Bar graphs show the results expressed as ratio of annexin V or PARP p85 and β-actin bands. Values are represented as mean ± SEM and $n = 6$/group. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control

Parkinson's disease, Huntington's disease, epilepsy, and other neurological disorders [47–55]. In this study, we found that phosphorylation of ERK1/2 and JNK is mediated through ROS generation, since pretreatment with ROS-inducing enzyme NOX inhibitor significantly attenuated phosphorylation of these proteins; however, this demonstrates no significant change in the expression of phosphorylated p38. Consistently, many reports have also shown that MAPKs are the downstream signaling molecules regulated by ROS [24, 56–58]. In addition, we demonstrated that ROS participates in upregulation of NF-kB; it could be via regulation of ERK1/2 and JNK pathways since pretreatment with ERK1/2 inhibitor, U0126, and JNK inhibitor, SP600125, significantly attenuated the expression of NF-kB. NF-kB is a well-known redox-regulated transcription factor that controls transcription of DNA, cytokine production, immune response, and other stress signals, including oxidative and hypoxic stresses associated with several physiological and pathological events [59–62]. Hsieh

et al. (2010) reported that TGF-β1-induced MMP-9 expression via NF-kB activation is mediated through ROS-dependent ERK1/2 and JNK cascades in RBA-1 cells [63]. However, different MAPK members are differentially involved in NF-kB activation in various cell types [32, 64, 65]. Mitochondrial dysfunction is the major cause of secondary injury in TBI-associated brain damage [66]. Mitochondrial injury leads to oxidative stress and subsequent apoptosis and decreased cellular energy production [67]. In our study, mitochondrial dysfunction could be one of the major causes of induction of oxidative stress since it is the major source of ROS [68]. ROS include free radicals, such as superoxide, hydroxyl radical, and singlet oxygen, as well as hydrogen peroxide, a non-radical species [44]. Glutamate-mediated excitotoxicity, perhaps, causes mitochondrial damage via high influx of intracellular Ca^{2+} during TBI [69]. Recently, we have reported that TBI causes high influx of Ca^{2+} that in turn leads to apoptosis and neuroinflammation via the activation of caspase-1 [18]. Excess of Ca^{2+} enhances ROS generation [70]. The interaction of oxidative stress and Ca^{2+} augments Ca^{2+}-induced tissue injury [71]. Both Ca^{2+} and ROS can directly activate caspases or calpains and facilitate the process of apoptosis during injury.

We concluded this study by linking TBI-induced phosphorylation of ERK1/2 and JNK and activation of NF-kB to cell death via apoptosis. Recent studies from our lab and others have demonstrated that oxidative stress, Ca^{2+} signaling, and neuroinflammation are major mechanisms contributing to post-traumatic neurodegeneration [18, 20, 21, 72, 73]. We have established the importance of oxidative signaling in TBI and proposed inflammatory and apoptotic cascades as effector mechanisms in mild TBI [16, 19–21, 44]. In this study, we proved that phosphorylation of ERK1/2 and JNK activates caspase-3, a major caspase involved in apoptosis [34], since pretreatment with ERK1/2 inhibitor, U0126, or JNK inhibitor, SP600125, significantly attenuated the expression of NF-kB. Interestingly, the combined treatment of U0126 and SP600125 further significantly reduced the expression of cleaved caspase-3 compared to U0126- or SP600125-treated cells. The results suggest that ERK1/2 and JNK, but not p38, has an important role in cell apoptosis by activating the caspase-3 enzyme. Recently, Rehman et al. (2015) demonstrated the critical role of active JNK and its activation of the intracellular signaling cascade that leads to synaptic dysfunction and neurodegeneration in TBI mouse brains [15]. Their major observations are with treatment of active JNK inhibitor, SP600125, markedly reduced several pathological events that included APP expression, Aβ production, Aβ-synthesizing enzymes, phosphorylation of tau proteins, neuroinflammation, BBB breakdown, neurodegeneration, and synaptic loss in TBI mouse brains. Similarly, Namura et al. (2001) demonstrated that the inhibition of the ERK pathway using U0126, a MAP kinase/ERK kinase-specific inhibitor, resulted in reduced brain damage [74].

Overall, the data of this study provides a description of the mechanisms of MAP kinase signaling pathway mediated neurodegeneration by activating ROS and inducing apoptosis in neuronal stretch injury and FPI animal models. This study focused on the importance of MAP kinase action in mTBI and thus provides a novel mechanism of neuronal apoptosis that may also potentially apply to other neurodegenerative diseases. Our present report encourages further work to determine the role of ERK1/2 and JNK in different cell types such as astrocytes, microglia, and endothelial cells in analyzing neuroprotection by exogenously treating different doses of MAP kinase inhibitors. These studies significantly extend our understanding of the pathobiology of TBI, providing unique insight into the rationale for the use of various therapeutic interventions using ERK1/2 and JUN inhibitors and analyzing their efficacy for the treatment of TBI-related neurological complications and other neurodegenerative diseases.

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Compliance with Ethical Standards

All experimental procedures involving animals were approved by the Seton Hall University Institutional Animal Care and Use Committee (IACUC). All procedures were performed in accordance with the strict guidelines of the 8th edition of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Conflict of Interest The authors declare that they have no conflict of interest.

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