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NADPH Oxidase Inhibition Improves Neurological Outcomes in Surgically-Induced Brain Injury

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Abstract

Neurosurgical procedures can result in brain injury by various means including direct trauma, hemorrhage, retractor stretch, and electrocautery. This surgically-induced brain injury (SBI) can cause post-operative complications such as brain edema. By creating a mouse model of SBI, we tested whether NADPH oxidase, an important reactive oxygen species producing enzyme, is involved in SBI using transgenic mice lacking gp91^{phox} subunit of NADPH oxidase (gp91^{phox} KO) and apocynin, a specific inhibitor of NADPH oxidase. Neurological function and brain edema were evaluated at 24 hours post-SBI in gp91^{phox} KO and wild-type littermates grouped into SBI and sham-surgery groups. Alternatively, mice were grouped into vehicle- and apocynin-treated (5mg/kg, i.p. 30 minutes before SBI) groups. Oxidative stress indicated by lipid peroxidation (LPO) was measured at 3 and 24 hours post SBI. The gp91^{phox} KO mice, but not the apocynin-treated mice showed significantly improved neurological scores. Brain edema was observed in both gp91^{phox} KO and wild-type groups after SBI; however, there was no significant difference between these two groups. Brain edema was also not affected by apocynin-pretreatment. LPO levels were significantly higher in SBI group in both gp91^{phox} KO and wild-type groups as compared to sham group. A trend, although without statistical significance, was noted towards attenuation of LPO in the gp91^{phox} KO animals as compared to wild-type group. LPO levels were significantly attenuated at 3 hours post-SBI by apocynin pretreatment but not at 24 hours post-SBI. These results suggest that chronic and acute inhibition of NADPH oxidase activity does not reduce brain edema after SBI. Long-term inhibition of NADPH oxidase, however improves neurological functions after SBI.

Keywords

Surgically-induced Brain Injury; NADPH Oxidase; Brain Edema; Oxidative Stress; Transgenic and Apocynin

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Introduction

Neurosurgical procedures can injure the susceptible normal brain tissue by various means including direct trauma, hemorrhage, retractor stretch, and electrocautery [1,9,10,21]. Routine neurosurgical operations are intrinsically linked to postoperative neurological deficits [5] and commonly result in postoperative complications such as brain edema and ischemia [6,17,23]. Our previous in-vivo studies using a rodent model have shown that brain edema following disruption of blood-brain barrier (BBB) plays an important role in pathophysiology of this surgically-induced brain injury (SBI) [12,13,18]. Pharmacological agents that attenuated the post-operative brain edema by preserving the blood brain barrier (BBB) integrity protected against SBI [12,13]. Alternatively, a known therapeutic agent such as erythropoietin increased brain edema by acutely increasing BBB permeability and was seen to worsen SBI [18].

Oxidative stress is a critical component of brain injuries resulting from different etiologies including ischemic, traumatic, hemorrhagic, and excitotoxic causes [3,4,15]. The role of oxidative stress in SBI, however, has not been addressed before. We hypothesized that oxidative stress contributes to brain edema and thus is important in etiopathogenesis of SBI.

NADPH oxidase is a major source of reactive oxygen species (ROS) and implicated in aforementioned brain injuries [15,19,22,24]. NADPH oxidase-induced ROS formation may lead to brain edema [22]. In the present study, we created a mouse SBI model to evaluate the effects of acute and chronic inhibition of NADPH oxidase using apocynin, a selective inhibitor [25] and knockout mice deficient in gp91^{phox}, a functional subunit of NADPH oxidase, respectively.

Method

Previously described wild-type (C57BL/6) and gp91^{phox} knockout (gp91^{phox} KO) mice that lack the membrane gp91^{phox} NADPH oxidase subunit [22] were obtained directly from Jackson laboratories (Harbor, ME, USA). All gp91^{phox} KO and age-matched wild-type mice were females and studied at 10–24 weeks of age. A total of 103 mice were randomly divided into the following groups: 1) gp91^{phox} KO – Sham 2) gp91^{phox} KO – SBI 3) Wild-type – Sham 4) Wild-type – SBI 5) Apocynin pretreated – SBI 6) Vehicle pretreated – SBI. Apocynin (5mg/kg) was administered intraperitoneally as a pretreatment 30 minutes before the surgery. Apocynin has been used in previous reports in different animal models to attenuate the oxidative stress contributed by ROS generation through NADPH oxidase [25]. DMSO was used as vehicle with final concentration less than 1%. All animals were sacrificed at 24 hours after SBI unless mentioned otherwise.

All surgical procedures were approved by the Loma Linda University Institutional Committee for Animal Care and Handling. Following anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p., the mice (approximately 20 grams) were placed in the prone position in a stereotaxic frame (Benchmark™) under a surgical operating microscope. After a midline incision was made by dissecting the skin and connective tissue, the periosteum was reflected to expose the right frontal skull. An operating square area (2mm edge) was identified on the frontal skull bone such that the left lower corner of the square was at the bregma (Figure 1A). The margins of the square were thinned out to translucency with a micro-drill without penetrating the skull. Using forceps, the piece of bone was gently lifted to expose a square window displaying the underlying right frontal lobe of the brain covered by dura. The dura was carefully incised with no. 22 needle to minimize bleeding and gently flipped over to expose the right frontal lobe. Using a bent flat blade (0.75 mm in width), two separate incisions were made leading away from the bregma along sagittal and coronal planes to sever an area of brain 2 mm lateral of sagittal and 1mm proximal of coronal sutures. The depth of the incisions

extended to the base of the skull. The sectioned brain mass were excised out and weighed approximately the same in all surgeries (data not shown). Intraoperative packing and saline irrigation was used to control bleeding. Hemostasis was confirmed by close observation after removal of packing. Subsequently, the scalp was sutured with 3–0 silk (Ethicon) on a reverse cutting needle. The procedure lasts approximately 25–30 minutes. Sham-operated animals underwent same surgical procedure, however, included only craniotomy and replacement of the bone flap without any dural incisions. Vital signs were monitored throughout the procedure. All mice were kept under heating lamp from to maintain the core body temperature 35–37°C until recovery.

Genotyping of gp91^{phox} subunit of NADPH oxidase was done on mice tails as described before [22] in order to confirm genetic deletion of the enzyme subunit in the mutant mice. Figure 1B shows that the band for NADPH gp91^{phox} subunit is located at the 240 bp in wild-type mice, but shifted to 195 bp in gp91^{phox} KO mice.

To evaluate brain edema, the animals were sacrificed under deep anesthesia at 24 hours after SBI. The brains were removed and divided into six parts: frontal ipsilateral, frontal contralateral, parietal ipsilateral, parietal contralateral, cerebellum and brain stem on ice. The cerebellum was used as an internal control for brain water content. The samples were weighed immediately (wet weight) on an electronic analytical balance (Denver Instrument, APX-60) to the nearest 0.001g and weighed again (dry weight) after drying in oven at 100°C for 48 hours. The percent of water content was calculated as [(wet weight – dry weight)/wet weight] x 100 % as described before [27].

Neurological scores were assessed 24 hours after SBI by an independent researcher blinded to the procedure using a modification of scoring (27 points) reported by Garcia et al [8]. Sensorimotor testing was graded on a scale of 0 to 3 each on spontaneous activity, symmetry of walking, head/neck movement when suspended on tail, forearm symmetry when suspended on tail, climbing ability, vibrissae response, side stroking response, pin prick pain response, ear pinch response. Neurological deficit scores were assigned as follows: 0, complete deficit; 1, definite deficit with some function; 2, decreased response or mild deficit; 3, no evidence of deficit/symmetrical responses.

To determine oxidative stress, the levels of lipid peroxidation product, malondialdehyde (MDA) were measured using an LPO-586 kit (Oxis Research, Portland, OR, USA) in brain samples after sacrifice as described earlier [15] MDA levels were measured in whole brains of sham and SBI groups in transgenic gp91^{phox}KO and wild type mice brains as well as apocynin- and vehicle- pretreated SBI mice brains. Briefly, brains were homogenized in 20 mM phosphate buffer (pH 7.4) containing 5 mM butylhydroxytoluene (BHT). The homogenates were centrifuged at 3000 g for 10 min at 4°C to remove large particles. The samples were allowed to react with a chromogenic reagent at 45°C for 60 min. The samples were then centrifuged at 15000 g for 10 min at 4°C and supernatants were measured at 586 nm. The level of MDA was calculated using a standard curve according to the manufacturer's instructions.

For obtaining histological data, deeply anesthetized animals were transcardially perfused with ice-cold 0.01 mol/L PBS (60 ml) pH 7.4 and then 4% formaldehyde solution at 24 hours after SBI. The brains were quickly removed, post-fixed in 4% formaldehyde solution at 4°C overnight, and immersed in 30% sucrose until they sank. The brains were then embedded, and horizontal sections (10 µm thick) were cut on to polylysine coated glass slides with the use of a cryostat as described previously [15,22]. Nissl's staining was performed as previously described using 0.5% cresyl violet [19].

Data are expressed as mean $\pm$ S.D. Statistical significance was verified by analysis of variance performed in one-way ANOVA for multiple comparisons. Unpaired t-test was used if only two groups were compared. Probability value of $p < 0.05$ was considered statistically significant.

Result and Discussion

The present study describes improvement of neurological outcomes following SBI in gp91^{phox} KO mice deficient in NADPH oxidase, a major source of ROS and oxidative stress. Sensorimotor deficits were observed (decrease in neurological scores) after SBI in gp91^{phox} KO as well as wild-type mice. The gp91^{phox} KO mice however showed significantly better neurological scores than the wild-type group (Figure 2). Similarly, apocynin (5mg/kg)- and vehicle-pretreated mice showed a decrease in neurological function following SBI. However, there was no significant difference between the two groups. ROS produced by NADPH oxidase have been suggested to contribute to many forms of brain injury [15,19,22,24]. Transgenic mice lacking in gp91^{phox} i.e the critical functional subunit of NADPH oxidase show lesser brain injury [22].

Both chronic and acute inhibition of NADPH oxidase did not reduce brain edema after SBI. Brain water content (brain edema) was significantly increased in ipsilateral frontal brain at 24 hours after SBI in both wild-type and gp91^{phox} KO mice as compared to their respective shams. There was, however, no significant difference in brain water content between the wild-type and gp91^{phox} KO mice (Figure 3). Both apocynin- and vehicle-pretreated mice also showed increased brain water content in ipsilateral frontal brain at 24 hours after SBI. However, there was no significant difference between the two groups (Figure 3). None of the other brain areas showed any significant brain edema.

Other possible mechanisms involving NADPH oxidase-induced neuronal/brain injury such as apoptosis [11,14] and inflammation [16,20,30] need to be investigated to evaluate the neuroprotective role of NADPH oxidase inhibition in SBI. Histological evidence by Nissl's staining showed a rim of dead neuronal cells surrounding the site of surgical resection (Figure 4A). Future studies will be aimed at studying whether apoptotic mechanisms play a role in this neuronal cell death especially in the transition zone i.e the brain area where the dead cells merge with the viable live cells (Figure 4B). It will be of interest if anti-apoptotic interventions will translate into neuroprotection after SBI.

The present study is the first instance of examining SBI and the postoperative complications in a transgenic mouse model. We successfully showed that SBI causes localized brain edema and resulting neurological deficits similar to earlier reports in rodent model [12,13,18]. The use of mouse model paves a way to study SBI using other transgenic animals. Additionally, we showed that oxidative stress occurs after SBI.

The effect of acute and chronic inhibition of NADPH oxidase on oxidative stress was assessed by measuring malondialdehyde (MDA), a product of LPO [15]. Acute inhibition with apocynin pretreatment significantly decreased oxidative stress at 3 hours after SBI, an effect which was lost at 24 hours after SBI (Figure 5B). Thus, apocynin may need to be administered in multiple dosages for therapeutic effects. Lack of pharmacological effect of apocynin at 24 hours may partly explain lack of improvement in neurological outcomes after SBI. Indeed, the acute Neuroprotective effect of apocynin was observed in cultured astrocytes and endothelial cells [28] and long term effect of apocynin conducted by using osmotic pumps was reported in a mouse sleep apnea model [29]. Further studies are needed to evaluate the pharmacodynamics of in-vivo apocynin administration. Oxidative stress i.e MDA levels increased significantly in both gp91^{phox} KO and wild-type mice 24 hours after SBI. The transgenic mice showed lower levels (though not significant) of MDA as compared to wild-type mice (Figure 5A). NADPH

oxidase is a major source of ROS; however other key sources such as cytochrome P450 and xanthine oxidase [2,26] may compensate for chronic NADPH oxidase inhibition and they need to be studied in SBI.

In conclusion, SBI increases oxidative stress and brain edema in the susceptible brain tissue. Chronic NADPH oxidase inhibition results in better neurological outcomes, however, does not affect the brain edema after SBI. The successful adaptation of SBI model from rodents to mice will help to study major pathways involved in SBI using transgenic animals.

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Figure 1.

Figure 1A shows a schematic drawing depicting rat brain cut along horizontal plane and showing the frontal lobe surgical injury in relation to the bregma. The two incisions are made leading away from the bregma along the sagittal and coronal planes 2 mm lateral and 1 mm proximal to the sagittal and coronal sutures respectively. Figure 1B shows genotyping of mice tails. The band for NADPH gp91^{phox} subunit is located at the 240 bp in wild-type mice, but shifted to 195 bp in gp91^{phox} KO mice.

Figure 2.

NADPH Oxidase Inhibition Improves Neurological Outcomes. Figure shows neurological scores after SBI in gp91^{phox} KO (n=16) as well as wild-type (n=15) mice as compared to their respective sham groups (n=21, n=19). The gp91^{phox} KO mice however showed significantly better neurological scores than the wild-type group. Apocynin (5mg/kg, n=12) and vehicle (n=14) pretreated mice showed a decrease in neurological scores after SBI. There was however, no significant difference between the two groups ($p>0.05$). Data is presented as mean $\pm$ S.D. * and # represents vs. wild-type and gp91^{phox} sham respectively and \$ represents vs. wild-type SBI. n numbers for each group are indicated in the figure.

Figure 3.

Figure shows brain water content (brain edema) in different areas of the brain 24 hours after SBI. Brain edema was significantly increased in ipsilateral frontal brain at 24 hours after SBI in both wild-type (n=11) and gp91^{phox} KO (n=17) mice as compared to their respective shams (n=8 each). There was, however, no significant difference in brain water content between the wild-type and gp91^{phox} KO mice. Both apocynin (5mg/kg, n=5), and vehicle (n=4) pretreated mice also showed increased brain water content in ipsilateral frontal brain after SBI. However, there was no significant difference between the two groups. All other areas of the brain did not show any significant brain edema ($p > 0.05$). Data is presented as mean $\pm$ S.D. * and # represents vs. wild-type and gp91^{phox} KO sham respectively. n numbers for each group are indicated in the figure.

Figure 4.

Nissl's staining done on horizontal brain sections 24 hours after SBI shows a rim of dead neuronal cells along the site of resection (Figure 4A). These dead neurons (arrows above dashed line) merged into the viable neurons (arrows below dashed line) farther from the site of resection. Higher magnification helped to identify a transition zone between the dead and viable neurons (Figure 5B). Scale bar represents 1mm in figure A and 200 μm in figure B.

Figure 5.

Determination of Oxidative Stress. MDA levels (picomoles per mg protein) used to assess lipid peroxidation increased significantly in both gp91^{phox} KO and wild-type mice 24 hours after SBI. The transgenic mice showed lower levels (though not significant) of MDA as compared to wild-type mice. Apocynin-pretreatment significantly decreased the MDA levels as compared to the vehicle-pretreated group at 3 hours after SBI, however, showed no significant change at 24 hours after SBI ($p > 0.05$). Data is presented as mean $\pm$ S.D. * and # represents vs. wild-type and gp91^{phox} KO sham respectively. \$ represents vs. vehicle-treated group. N=5 in each group.