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Role of Gap Junctions in Early Brain Injury Following Subarachnoid Hemorrhage

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Abstract

Gap junction inhibition has been demonstrated to reverse the vascular contraction that follows experimental subarachnoid hemorrhage. This study hypothesizes that the use of established gap junction inhibitors: octanol and carbenoxolone, to interrupt cell to cell communication will provide neuroprotection against early brain injury after SAH. The filament perforation model of SAH was performed in male Sprague–Dawley rats weighing between 300 and 380g. Octanol (260.46mg or 781.38 mg/kg), carbenoxolone (100 mg/kg), or vehicles were given via intraperitoneal injection 1 hour after SAH. Neurologic deficits and cerebral apoptosis were assessed 24 and 72 hours after SAH. In addition, Western blot analysis was performed to confirm the *in vivo* inhibition of CNS gap junctions. The administration of octanol and carbenoxolone both failed to attenuate the neurological deficits induced by SAH, and they did not reduce neuronal apoptosis. Additionally, carbenoxolone increased post SAH mortality and exacerbated SAH induced apoptosis. Despite previous studies that show gap junction inhibitors reverse vasospasm following experimental SAH, they failed to improve clinical outcomes or provide neuroprotection in this study.

Keywords

Gap Junction; Octanol; Carbenoxolone; Connexin 43; Subarachnoid Hemorrhage; Early Brain Injury

1. Introduction

Stroke is the third leading cause of death, and the leading cause of major disability in the United States (Mackay J 2004). Five to seven percent of all strokes result from the rupture of cerebral aneurysms resulting in subarachnoid hemorrhage (SAH), and this form of cerebral vascular disease affects 1/10,000 people annually (Broderick et al. 1992; Kassell et al. 1985). SAH carries significant morbidity and mortality, with 40% of patients dying within one month, and 33% of survivors harboring significant neurologic deficits (MCCORMICK & Nofzinger 1965; Schievink et al. 1995).

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Delayed cerebral vasospasm has traditionally been recognized as the most treatable cause of morbidity and mortality from SAH, however, evidence is mounting that the physiological and cellular events of early brain injury following aneurysm rupture make significant contributions to patient outcomes (Broderick et al. 1994). Early brain injury in aneurysmal SAH is the result of physiological derangements such as increased intracranial pressure (ICP), decreased cerebral blood flow (CBF), as well as the direct toxicity to the central nervous system (CNS) from blood in the subarachnoid space (Ostrowski, Colohan, & Zhang 2006). These events lead to the early development of edema, oxidative stress, inflammation, apoptosis, and infarction (Cahill, Calvert, & Zhang 2006; Endo et al. 2007; Ferguson & Macdonald 2007; Kamiya, Kuyama, & Symon 1983; Kaynar et al. 2005; Kubota et al. 1993; Laszlo, Varga, & Doczi 1995; Mathiesen & Lefvert 1996; Polidori et al. 1997; Prunell et al. 2005; Yatsushige et al. 2007). Experimental models have shown significant white matter injury and neuronal death (Cahill, Calvert, & Zhang 2006), progression of apoptosis (Prunell, Svendgaard, Alkass, & Mathiesen 2005), which seemed mediated through the activation of a JNK/cJun (Yatsushige, Ostrowski, Tsubokawa, Colohan, & Zhang 2007), and other classical apoptotic pathways following SAH (Cahill, Calvert, & Zhang 2006). Studies also demonstrate that the inhibition of apoptotic pathways following SAH not only reduced cellular death, but also resulted in a significant improvement in functional outcome (Cahill, Calvert, & Zhang 2006; Yatsushige, Ostrowski, Tsubokawa, Colohan, & Zhang 2007).

Gap junctions are conductive channels connecting the cytoplasmic domains of adjacent cells (Bennett & Goodenough 1978; Naus & Bani-Yaghoub 1998). They are formed in cell membranes and are composed of an array of connexin proteins that create a central channel allowing for direct communication with the adjacent cell. These channels are known to connect adjacent neurons as well as form connections among astrocytes (Nagy & Rash 2000). CNS gap junctions in the developing brain play a major role in the control of the regulated apoptosis that shapes adult CNS development (Cusato et al. 2003; Naus & Bani-Yaghoub 1998), however, the roles of gap junctions in the adult brain under normal conditions is not clear. Recent studies of their function under pathological conditions are providing evidence that gap junction communication may allow for the transmission of apoptotic and necrotic cell signals, amplifying the extent of injury (Frantseva et al. 2002; Lin et al. 1998). This mechanism for the transmission of cell death signals may make significant contributions to poor outcomes in several forms of CNS injury. Models of focal cerebral ischemia demonstrate that the secondary expansion of infarction is reduced by blocking gap junctions (Rawanduzy et al. 1997). Experiments using models of global cerebral ischemia in both adult and neonatal rodents have shown that the systemic administration of gap junction inhibitors prevented neuronal cell death in the CNS (de Pina-Benabou et al. 2005; Perez Velazquez et al. 2006; Rami, Volkmann, & Winckler 2001).

It is therefore imperative to test the efficacy of gap junction inhibitors on neurological outcome following SAH. This study hypothesizes that the systemic administration of the gap junction inhibitors octanol and carbenoxolone following SAH will prevent cerebral apoptosis and lead to improved neurological outcomes.

2. Results

Mortality Rate and Total Number of Animals for Analysis following Exclusion

In summary, a total of 146 animals underwent surgery. Fifteen SHAM and 131 SAH surgeries were performed. Twenty animals died from SAH before the administration of either vehicle or treatment, leaving 111 SAH animals that received treatment. Seventeen of the 111 SAH animals that received treatment died before their sacrifice at either 24 or 72 hours after SAH. SHAM groups had no mortality. Overall SAH induced a significant mortality (37 of 131 [28%]) compared to SHAM surgery ($P < 0.05$ vs SHAM). Once animals survived the hour preceding

the administration of either vehicle or treatment, there were no differences in mortality between vehicle and octanol treated SAH groups ($P>0.05$ Gehan-Breslow statistic for the survival). However, SAH groups had significantly higher mortality following the administration of carbenoxolone, suggesting an induced toxicity ($P<0.05$ SAH+carbenoxolone vs SHAM and SAH+octanol groups on Gehan-Breslow statistic for the survival). Of the 94 SAH animals that survived until sacrifice, 28 were excluded from neurological and molecular analysis for having mild subarachnoid hemorrhage in the basal cisterns in accordance with the data from Sugawara et al. (Sugawara et al. 2008). Following mortality and exclusions, 15 SHAM and 66 SAH animals were included in the analysis of outcomes at 24 or 72 hours after SAH. Note: Only 2 animals survived until 72 hours for SAH+ carbenoxolone 100 mg/kg group and were subsequently excluded from further analysis due to small sample size.

Experimental SAH Produced Equal SAH Severity Among All Tested Groups

All SAH groups included in the mortality analysis and those evaluated for outcomes at 24 and 72 hours had no significant differences in their bleeding scale (Figure 1A and 1B), indicating that the degree of injury was equal among all groups. Although there is a trend for less subarachnoid blood in the animals evaluated at 72 hours this did not reach statistical significance. This phenomenon most likely represents the clearing of blood from the subarachnoid space over time.

Gap Junction Inhibition Failed to Ameliorate Neurological Deterioration following Experimental SAH

Modified Garcia scoring and beam balance tests performed on the animals subjected to SAH showed significant neurological deficits in comparison to SHAM groups at 24 and 72 hours (Figures 2A-B) ($P<0.05$ for all SAH+vehicle groups vs SHAM at both 24 and 72h). Treatment with octanol at 260.46 mg/kg and 781.38 mg/kg, and carbonoxolone at 100 mg/kg failed to improve neurological scores in comparison to vehicle treated groups ($P>0.05$ for SAH+ octanol 260.46 mg/kg, SAH+ octanol 781.38 mg/kg, and SAH+carbenoxolone 100 mg/kg vs SAH +vehicle at 24 and 72h).

Gap Junction Inhibitors Fail to Reduce Apoptosis Induced by Experimental SAH

Early brain injury peaked at 24 hours after SAH as indicated by significant increases in cerebral apoptosis following SAH (Figure 3A). Cell death markers were observed at 24 hours, but dissipation by 72 hours. This temporal profile for apoptosis following SAH has been seen in previous studies with the same ICA puncture model of experimental SAH (Cahill et al. 2006; Cahill et al. 2007; Sugawara et al. 2009). Octanol failed to attenuate the cellular death induced by SAH, while carbenoxolone exacerbated cellular death at 24 hours (Figure 3A).

Octanol Administration Inhibited Gap Junctions

The intraperitoneal administration of octanol inhibited functional gap junctions (Figure 3B). Western blot analysis demonstrated a significant increase in the serine 368 phosphorylation of connexin 43 following the administration of octanol in both low and high doses ($P<0.05$ vs SHAM). This finding is consistent with the presence of inhibited gap junctions following the administration of octanol.

3. Discussion

This is the first experiment to test the efficacy of gap junction inhibitors in early brain injury following experimental subarachnoid hemorrhage. Our study is different from the previous reports in that we used a filament puncture rat model to induce early brain injury, and second we evaluated neurological functions of animals after gap junction inhibitor treatment. Our

results showed that gap junction function does not seem to be directly involved in the pathogenesis of early brain injury after SAH.

Hong et al previously demonstrated the efficacy of gap junction inhibitors in reversing the contraction of cerebral arteries in rabbits (Cahill, Calvert, Solaroglu, & Zhang 2006; Cahill, Calvert, Marcantonio, & Zhang 2007; Hong et al. 2008; Hong et al. 2009). However, in these experiments a cisternal injection model of SAH was used, the drugs were administered intracisternally, and results were based on ex vivo measurements of basilar arteries. Even though gap junction inhibition may prevent vasospasm, the reversal of vascular spasm following SAH has not proven to correlate with improved clinical outcomes (Macdonald et al. 2008), and studies linking vasospasm to clinical outcome have come under recent scrutiny (Ferguson & Macdonald 2007; Fisher, Roberson, & Ojemann 1977; Nolan & Macdonald 2006; Rabinstein et al. 2004). Nimodipine, the only FDA approved pharmacological agent for the treatment of aneurysmal SAH patients (Petruk et al. 1988) does not reduce radiographic vasospasm despite its associated improved outcomes (Haley, Jr., Kassell, & Torner 1993). Evidence from the Clazosentan trials (Macdonald, Kassell, Mayer, Ruefenacht, Schmiedek, Weidauer, Frey, Roux, & Pasqualin 2008), which failed to show improved outcomes with significant reversal of vasospasm, may put a new emphasis on this direction of research, especially on early brain injury. Our experiment takes the preliminary investigation by Hong et al (2009) a step further by using gap junction inhibitors in a filament model of SAH recognized as more closely simulating true aneurysmal SAH (Bederson, Germano, & Guarino 1995). We found that gap junction inhibition failed to prevent early brain injury in this SAH model.

Neurological functional evaluation is an essential step in the assessment of a treatment effect. Improved outcome of the patient is the ultimate goal, regardless of the mechanism, and it is essential to include this variable in translational research. Preceding in vivo experiments implementing the administration of gap junction inhibitors in models of CNS injury did not evaluate neurological outcome (de Pina-Benabou, Szostak, Kyrozis, Rempe, Uziel, Urban-Maldonado, Benabou, Spray, Federoff, Stanton, & Rozental 2005; Perez Velazquez, Kokorovtseva, Sarbaziha, Jeyapalan, & Leshchenko 2006; Rami, Volkmann, & Winckler 2001). A possible action of gap junction inhibitors on neurological outcome or mortality after SAH was not studied (Hong, Wang, Wang, & Wang 2008). As mentioned above, the occurrence of cerebral vasospasm and the incidence of neurological deterioration following SAH do not always correlate (Claassen et al. 2001; Ferguson & Macdonald 2007; Shimoda et al. 2001), and a recent review of the literature demonstrates that the link between angiographic vasospasm and poor outcome is only weakly supported by the literature (Nolan & Macdonald 2006). Clinical trials have demonstrated that reducing angiographic vasospasm by as much as 65% resulted in only mild reductions in delayed neurological deficits, and failed to result in improved functional outcome (Vajkoczy et al. 2005). Our experiment demonstrated the significant neurological deficits in this SAH model which is consistent with previous experiments (Cahill, Calvert, Solaroglu, & Zhang 2006; Endo, Nito, Kamada, Yu, & Chan 2007; Sugawara, Jadhav, Ayer, Chen, Suzuki, & Zhang 2009). We failed however to observe any neurological functional improvement after using gap junction inhibitors in this study.

The potential mechanisms of gap junction in early brain injury after SAH are investigated. Apoptotic cellular death following experimental aneurysmal SAH has been well characterized (Cahill, Calvert, Marcantonio, & Zhang 2007; Endo, Nito, Kamada, Yu, & Chan 2007; Prunell, Svendgaard, Alkass, & Mathiesen 2005), and its significance in clinical outcomes has recently come into focus, implicating its role in the delayed neurological deficits (DNDs) following aneurysmal SAH (Ayer & Zhang 2008; Cahill, Calvert, & Zhang 2006; Ostrowski, Colohan, & Zhang 2006). However, octanol failed to demonstrate any effect on post SAH apoptosis. Additionally, carbonalexone demonstrated increased cellular death following SAH, as well as increased mortality.

From the obtained results, it seems SAH is unique when compared with other cerebral ischemic disorders regarding a role of gap junctions. Previous experiments in focal ischemia and neonatal hypoxia-ischemia have shown gap junction inhibitors to be protective against neuronal cell death (de Pina-Benabou, Szostak, Kyrozis, Rempe, Uziel, Urban-Maldonado, Benabou, Spray, Federoff, Stanton, & Rozental 2005; Perez Velazquez, Kokarovtseva, Sarbaziha, Jeyapalan, & Leshchenko 2006; Rami, Volkmann, & Winckler 2001). This difference may arise from the molecular events that initiate cell death in each of these models. In focal ischemia, the theory of protection provided by gap junction inhibitions is the blockage of cell death signals traveling intracellularly from cell to cell that originate in the area of infarction (Lin, Weigel, Cotrina, Liu, Bueno, Hansen, Hansen, Goldman, & Nedergaard 1998; Rawanduzy, Hansen, Hansen, & Nedergaard 1997). Blocking the transmission of these signals should in theory spare cells in the ischemic penumbra, and reduce the extent of infarction. In global ischemia, the mechanism of gap junction protection is less clear, as theories involving the inhibition of apoptosis, electrical silencing, and anti-oxidant effects have been proposed (de Pina-Benabou, Szostak, Kyrozis, Rempe, Uziel, Urban-Maldonado, Benabou, Spray, Federoff, Stanton, & Rozental 2005; Perez Velazquez, Kokarovtseva, Sarbaziha, Jeyapalan, & Leshchenko 2006; Rami, Volkmann, & Winckler 2001). Aneurysmal SAH is distinct from both focal and global ischemia in many ways, but the exsanguination of blood and the release of oxyhemoglobin (oxyHb) into the subarachnoid space highlights why inhibitors of cell to cell communication may fail. In SAH the toxic signals are extracellular, perhaps minimizing the importance of intracellular cell to cell apoptotic signals. For example the liberation of (oxyHb) into the cerebrospinal fluid following SAH is a major producer of superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) as it undergoes auto-oxidation to methemoglobin (Asano 1999). Many studies demonstrate the oxidizing capacity of hemoglobin on lipid membranes, proteins, and DNA (Goldman et al. 1998; Rogers et al. 1995; Sarti et al. 1994), and subarachnoid hemolysate also increases cytochrome c mediated DNA fragmentation and apoptosis in mouse brains (Matz, Fujimura, & Chan 2000).

The potential limitations facing this translational experiment include the possibility that gap junctions are effective neuroprotects in SAH, but suboptimal dosing was chosen. However, the 781.38mg/kg dose of octanol was chosen based on similar dosages that were found to be effective in rodent models of ischemic CNS injury, and an attempt at eliciting a dose response was made by choosing a dosage that was 1/3 of previously effective dosages. A study of the pharmacokinetics of octanol in this rat model of SAH that may able to define a dosage regimen that would provide a more effective CNS concentrations of the drug, however the selection of dosages based on reports of efficacy in other CNS diseases is a reasonable initial approach. Investigating why previously successful dosages of gap junction inhibitors for other CNS diseases failed in SAH may lead to a better understanding of the inherent role of CNS gap junctions. Additionally, the therapeutic window for the effective administration of gap junction inhibitors may have been sub-optimal. The therapeutic window for early brain injury following SAH is a complex issue. In most cases, the earlier a neuroprotective treatment can be administered the better the outcome, with pre-treatment many times offering better results than any post injury intervention. However, the timing and duration of treatment is often dependent on the type of pharmacological intervention being used. In the case of VEGF and MMP, inhibition is only protective for a specific duration following injury, and should be stopped shortly after the acute insult in order to preserve the reparative effects of MMPs and VEGF in the more subacute phases of injury (Mandal et al. 2003; Zhang et al. 2000). Previous experiments seem to suggest that early treatment, or even pretreatment with gap junctions may be most effective (de Pina-Benabou, Szostak, Kyrozis, Rempe, Uziel, Urban-Maldonado, Benabou, Spray, Federoff, Stanton, & Rozental 2005; Frantseva, Kokarovtseva, Naus, Carlen, MacFabe, & Perez Velazquez 2002; Perez Velazquez, Kokarovtseva, Sarbaziha, Jeyapalan, & Leshchenko 2006), however, we chose to administer gap junction inhibitors one hour after

SAH in order to simulate the delay that exists between the presentation of symptoms and arrival to the hospital for treatment.

In summary this study demonstrated that the administration of octanol following experimental aneurysmal SAH effectively inhibited CNS gap junctions, but failed to inhibit neuronal apoptosis, or improve neurological outcomes 24 and 72 hours following SAH. It is the first study to investigate gap junction inhibition in early brain injury following experimental SAH, and these negative findings highlight the importance of translational research that closely parallels clinical practices.

4. Experimental Procedure

Experimental Design

This experiment was a controlled *in vivo* laboratory study taking place in an animal research laboratory utilizing Male Sprague Dawley outbred rats (Harlan). All procedures and experiments were approved by the Institutional Animal Care and Use Committee of Loma Linda University. Animals for each experimental group were randomly chosen from purchased batches of animals; all purchased animals were included in the study. The treatments following surgery were randomly assigned to each rat by a researcher independent of the surgeon. Neurological scores were performed by an independent researcher who was blinded to the surgery performed (SHAM versus SAH), as well as the treatment administered (vehicle versus octanol versus carbenoxolone). Brain water content measurements and Western Blot analysis were performed by researchers blinded to the experimental groups.

Experimental Groups and Quantification of SAH

Prior to surgery animals were randomly assigned to one of five groups: (1) SHAM surgery plus the administration of vehicle solution (triglyceride oil), (2) SAH surgery + vehicle solution (triglyceride oil), (3) SAH + 260.46 mg/kg of octanol, (4) SAH + 781.38 mg/kg of octanol, or (5) SAH + 100 mg/kg of carbenoxolone. Animals were then randomly assigned to be sacrificed at 24 or 72 hours after SAH. Following sacrifice, the severity of the SAH was evaluated in a blinded manner as described by Sugawara et al (Sugawara, Ayer, Jadhav, & Zhang 2008). Briefly, the SAH grading system is as follows: the basal cisterns are divided into 6 segments and each segment is allotted a grade from 0-3 depending on the amount of subarachnoid blood clot in the segment; Grade 0: no subarachnoid blood, 1: minimal subarachnoid blood, 2: moderate blood clot with recognizable arteries, 3: blood clot obliterating all arteries within the segment. The animals received a total score ranging from 0 -18 after adding the scores from all 6 segments (Figure1). Animals with a bleeding scale of less than 8 were excluded from analysis based on previous reports indicating that mild SAH fails to result in significant neurological decline, and failure to demonstrate the efficacy of neuroprotectants (Sugawara, Ayer, Jadhav, & Zhang 2008). This randomization process continued until there were at least 7 animals per group at both 24 and 72 hours after SAH.

Induction of SAH

SAH in rats was experimentally induced using the endovascular perforation model as previously described (Bederson, Germano, & Guarino 1995). Briefly, general anesthesia was induced with isoflurane 0.5-5% followed by atropine (0.1 mg/kg s.c.). After intubation the animals were ventilated with an animal ventilator (Harvard Apparatus), and anesthesia was maintained by via the titration of isoflurane anesthetic 0.5-5% in 70% medical air with 30% oxygen. A heating pad and heating lamp were used to maintain the rectal temperature at $36.0 \pm 0.5^{\circ}\text{C}$ during surgery, and while the animal recovered from anesthesia post operatively. After exposing the left common carotid artery (CCA), external carotid artery (ECA), and ICA through a midline skin incision, the ECA was ligated, cut, and shaped into a 3mm stump. A

sharpened 4-0 nylon suture was advanced rostrally into ICA from the ECA stump until resistance was felt (15 A 18mm from common carotid bifurcation), and then pushed 3 mm further to perforate the bifurcation of the anterior cerebral and middle cerebral arteries. Immediately after puncture the suture was withdrawn and the ICA was re-perfused. Operative procedures were exactly same for the sham group, except that the suture was removed once it was introduced ~15mm from the carotid bifurcation. The incision was then closed and rats were housed individually following their recovery from anesthesia. All rats received 2mL normal saline subcutaneously to prevent dehydration, and animals had free access to food and water until euthanization.

Drug Administration

One hour after the procedure, treatment groups received either a high (6 mmol/kg) or low (2 mmol/kg) dosage of octanol (Sigma), or a 100 mg/kg dose of carbenoxolone via an intraperitoneal injection. High dosage octanol (781.38 mg/kg) was selected on guidance from previous literature which demonstrated that similar dosages reduced infarct volume and decreased neuronal cell death following ischemia (Rami, Volkmann, & Winckler 2001; Rawanduzy, Hansen, Hansen, & Nedergaard 1997). The low dosage (260.46 mg/kg) was chosen to demonstrate dosage dependent effects while avoiding possible toxicities. All octanol dosages were dissolved in triglyceride oil as previously described, and injected in a volume of 3.5ml/kg (Martin & Handforth 2006). The animals sacrificed at 72 hours also received treatment at 24 and 48 hours after surgery. Carbenoxolone was dissolved in 0.9% saline at a dose of 100 mg/kg, and injected in a volume of 3.5ml/kg; this preparation is similar to the dosage used to demonstrate CNS penetration following intra-peritoneal delivery (Jellinck et al. 1993), and provided neuroprotection in a rodent model of neonatal hypoxia (de Pina-Benabou, Szostak, Kyrozis, Rempe, Uziel, Urban-Maldonado, Benabou, Spray, Federoff, Stanton, & Rozental 2005). Animals sacrificed at 72 hours received maintenance doses of 50 mg/kg carbenoxolone every twelve hours prior to sacrifice. The vehicle solution used for the SHAM+Vehicle and SAH+Vehicle groups was triglyceride oil. Although 0.9% saline was used to dissolve carbenoxolone, SHAM+saline and SAH+saline groups were not tested given previous experiments demonstrating the inert effects of 0.9% saline on neuroprotection in this model of SAH (Ayer et al. 2008).

Neurological Scoring

All neurological scores were evaluated 24 or 72 hours after SAH, and conducted by an individual blinded to the conditions of the experiment. The first neurological test performed to evaluate the sensorimotor deficits following SAH was an 18 point scoring system that is a modification of the test reported by Garcia et al (Garcia et al. 1995). Additional testing included an evaluation of the animals' performance on a beam test at 24 or 72 hours after SAH. This test is a modified adaptation of that used by Colombel et al, and was scored using a 4 point system (Colombel, Lalonde, & Caston 2002). Briefly, the rats were placed on a wooden horizontal rod (50 cm in length and 2.5 cm in diameter) covered with masking tape in order to provide firm gripping. The beam was divided into five segments of 10 cm and placed 60 cm above a padded platform. At the beginning of the test, the animal was placed on the middle of the rod, its body axis perpendicular to the beam's longitudinal axis. Latencies before falling with a 60 second cut off, and the distance walked were measured. Three trials were performed separated by 5 minutes. The highest score of the three trials was recorded as the rats' performance. Points were awarded as follows: 4 points for walking a distance of greater than 20cm, 3 points for walking a distance of at 10-20cm, 2 points for walking more than 10cm but falling before 60 seconds, 1 point for remaining on the beam for 60 seconds but not moving more than 10cm, 0 points for falling from the beam without traveling at least 10cm.

Apoptosis ELISA

For quantification of apoptotic cell death, we used a commercial enzyme immunoassay to determine cytoplasmic histone-associated DNA fragments (Roche Molecular Biochemicals, Indianapolis, IN, USA) from cortical brain tissue as previously described (Endo, Nito, Kamada, Yu, & Chan 2007). In summary, rats were placed under deep anesthesia (isoflurane 5% in 70/30% medical air/oxygen) before transcardiac perfusion of the brain with 180cc of ice cold phosphate buffered saline (PBS). The rats were then decapitated and the brains were removed, dissected into cortical, basal ganglia, cerebellar, and brainstem sections before being stored at -80° C. The protein from the left cortices of each rat was extracted using RIPA lysis buffer (Santa Cruz Biotechnology, USA). A cytosolic volume containing 20 µg of protein was used for an enzyme-linked immunosorbent assay following the manufacturer's protocol.

Western Blotting Analysis

Connexin 43 phosphorylation was used to confirm the inhibition of gap junctions in vivo. Connexin 43 is one of the most abundant gap junction proteins in vertebrates and is major component of CNS gap junctions (Dermietzel et al. 1989). The phosphorylation state of this protein strongly corresponds to the presence or absence of functional gap junctions (Solan & Lampe 2007). Several in vitro studies demonstrate that the phosphorylation of the serine 368 sit on connexin 43 corresponds with decreased cell-to-cell communication via gap junctions (Lampe et al. 2000). Therefore, we used Western blotting to confirm gap junction inhibition following the administration of octanol by measuring connexin 43 phosphorylation at serine 368 from 4 randomly chosen samples of rats' cerebral cortices 24 hours after SAH.

Western blot analysis was performed as described previously (Ostrowski, Colohan, & Zhang 2005). Animals were euthanized at 24 h after SAH. After transcardiac perfusion of the brain with 180cc of ice cold PBS, brains were removed and stored at -80°C immediately until analysis. Protein extraction from left hemisphere (ipsilateral to perforation) was obtained by gently homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Inc, sc-24948) followed by centrifuging at 14,000g at 4°C for 20 min. The supernatant was used as whole cell protein extract and the protein concentration was determined by using a detergent compatible assay (Bio-Rad, DC protein assay). Equal amounts of protein (50 µg) were loaded on a SDS-PAGE gel. After being electrophoresed and transferred to a nitrocellulose membrane, the membrane was then blocked and incubated with the primary antibody overnight at 4°C. The primary antibody was rabbit polyclonal anti-phospho-connexin-43 (Cell signaling, #3511S, 1:1000). Nitrocellulose membranes were incubated with secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham Biosciences, Arlington Heights, IL) and exposed to films. Blot bands were quantified by densitometry with Image J software (Image J 1.40g, NIH). β -Actin (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) was blotted on the same membrane as a loading control, and phosphorylated connexin was calculated as a ratio of β -actin.

Statistical Analysis

The data are expressed as mean $\pm$ standard error of the mean. Statistical differences between the various groups were assessed with a one-way analysis of variance (ANOVA) with Holm-Sidak posthoc analysis. Mortality was analyzed using the Gehan-Breslow statistic for the survival. A value of $P < 0.05$ was considered statistically significant. The statistical power of the one way ANOVA testing for Garcia scoring, Beam Balance, and the Apoptosis Elisa Assay was 0.827, 0.976, and 0.916 ($\alpha = 0.050$) respectively.

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Abbreviations

SAH	subarachnoid hemorrhage
CBF	cerebral blood flow
DNDs	delayed neurological deficits
oxyHb	oxyhemoglobin
$O_2^{\bullet}$	superoxide anion
H_2O_2	hydrogen peroxide

FIGURE 1.

Distribution of SAH Severity Among Experimental Groups. A) Demonstrating equivalent SAH severity between all groups included in the mortality analysis. Inset picture shows representative SAH from SHAM to Grade 16. B) Demonstrating that animals evaluated for neurological function, apoptosis, and connexin phosphorylation all have equivalent SAH severity. There are no statistically significant differences between the groups in either A or B. A trend for decreased subarachnoid blood at 72 hour does not reach significance (B). N numbers for each group are indicated at the top of each column. Error bars equal standard error.

FIGURE 2.

Neurological Outcomes Following Experimental SAH. A) Experimental SAH decreases animal performance in beam balance (A) and modified Garcia scoring (B) at 24 and 72 hours following SAH (* $P < 0.05$ vs SHAM for all groups). Treatment has no effect on performance at 24 or 72 hours following injury. N numbers for each group are indicated at the top of each column. Error bars equal standard error.

FIGURE 3.

Molecular Effects of Gap Junction Inhibition Following Experimental SAH. A) Whole brain apoptosis increases significantly 24 hours following SAH (* $P < 0.05$ vs SHAM for all groups). Carbenoxolone demonstrates increased whole brain apoptosis versus the untreated SAH group ($tP < 0.05$ carbenoxolone vs SAH+vehicle). B) Western blot analysis shows significantly increased connexin phosphorylation following the administration of the gap junction inhibitor octanol (* $P < 0.05$ 260.46mg/781.38mg octanol vs SHAM and SAH+vehicle), suggesting that treatment effectively inhibits functional gap junctions. Inset picture demonstrates representative Western blots. Expression levels of phosphorylated connexin 43 are expressed as a ratio of β -actin levels for normalization. N numbers for each group are indicated at the top of each column. Error bars equal standard error.