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Research Report

Protective mechanism of sulindac in an animal model of ischemic stroke ☆

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ABSTRACT

Background and Purpose: The present study analyzed whether administration of sulindac, a non-steroidal anti-inflammatory drug (NSAID) would prevent, attenuate or repair ischemia induced brain injury and reverse functional impairment in a focal ischemia model of stroke. *Methods:* Male Sprague-Dawley rats (weight 250–300 g) were subjected to middle cerebral artery occlusion (MCAO). Sulindac was given 2 days before and 24 h after ischemia at 0.2 mg/day with daily injections until sacrifice on day 3 or day 11. Infarct size was measured by TTC staining and western immunoblot was employed. *Results:* TTC analysis of brain slices indicated a decrease in infarct size in sulindac treated animals. Western blot results indicated that sulindac induced expression of Hsp 27, a marker of cell stress, in the ischemic penumbra and core on days 3 and 11. Hsp 27 is important as a protective molecular chaperone. Increases were also found in the protective molecules Akt and Bcl-2 in the ischemic penumbra and core following sulindac administration. *Conclusion:* Our data indicate that administration of sulindac results in decreased infarct size and that there is a central role for the molecular chaperone Hsp 27, the pro-survival kinase Akt and the anti-apoptotic component Bcl-2 in mediating these protective effects.

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1. Introduction

Sulindac is a non-steroidal anti-inflammatory drug (NSAID) that is capable of inhibiting cyclo-oxygenases (COX) 1 and 2 (Vane, 1971). In addition to its known anti-inflammatory activity there have been numerous studies in recent years on the ability of sulindac and its metabolites to act as potential anti-cancer agents, based on their ability to slow the progression of colorectal polyps to colon cancer, as well as their ability to kill colon and other cancer cells (Marchetti et al., 2009; Taketo, 1998).

Mammalian cells can respond to a variety of stresses such as heat, cold, oxidative stress, metabolic disturbance, and environmental toxins through necrotic or apoptotic cell death, while increased expression and phosphorylation of heat shock proteins such as Hsp 27 can protect cells against cellular stress. Heat shock proteins (Hsp) commonly exhibit molecular chaperone activity and also interact with a wide variety of proteins to exert specific effects. In the central nervous system, Hsp are induced in response to many injuries including stroke, neurodegenerative disease, epilepsy, and trauma. Hsp are highly conserved and under physiological conditions act as molecular chaperones and/or have anti-apoptotic activities. Two of the major heat shock proteins in the brain are the 70 kDa Hsp (Hsp 70) and the 27 kDa Hsp (Hsp 27) (Stetler et al., 2009). Over expression of Hsp 27 has been shown to reduce cortical damage after cerebral ischemia (Van der Weerd et al., 2010). Hsp 27 is characterized by its inducibility in both glial cells and neurons following a wide range of noxious stimuli including ischemia, epileptic seizure and hyperthermia (Van der Weerd et al., 2010).

Recent studies suggest that sulindac protects normal cells against oxidative damage. Previous studies on the heart suggested that sulindac protection against ischemic damage occurs through an ischemic preconditioning mechanism.

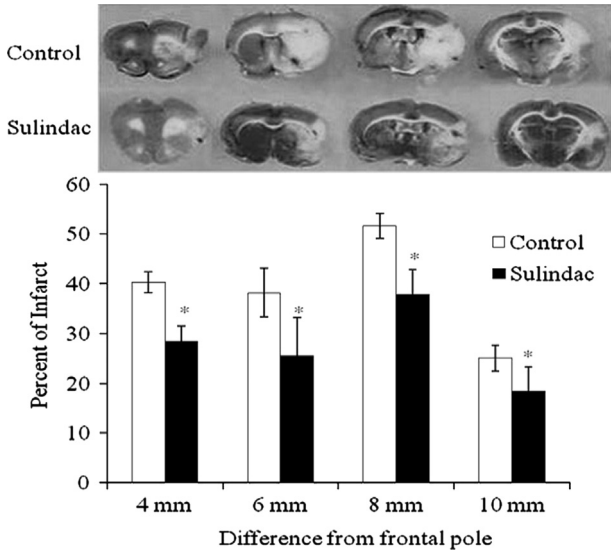

Fig. 1 – TTC analysis of brain slices indicated a decrease in infarct size in sulindac treated animals at 4 mm, 6 mm, 8 mm and 10 mm from the anterior pole ($P<0.01$; 2 way ANOVA). Data represent infarct volume as percent of ipsilateral hemisphere volume and values are means ± SE of 10 experiments for MCAO and MCAO plus Sulindac.

Sulindac was found to induce iNOS and Hsp 27 in a protein kinase C (PKC) dependent manner. It has been widely proposed that compounds that could precondition cells to oxidative stress may have important therapeutic value, since oxidative damage appears to play a major role in age related diseases (Moench et al., 2009).

In the current study we employed a rodent model of transient focal ischemia because this is very similar to human stroke in terms of pathophysiology. The middle cerebral artery (MCA) is the most commonly affected blood vessel in human occlusive/ischemic stroke (Virtanen et al., 2003) and the MCA is the artery most commonly targeted in rodent stroke models (Macrae, 2011).

In the present study, we have examined the protective effect of sulindac elicited by ischemia/reperfusion in the rat brain subjected to MCA occlusion (MCAO) and we provide evidence that sulindac is highly protective and exerts this pro-survival effect through pathways associated with pharmacological preconditioning.

2. Results

2.1. *Analysis of TTC staining*

Analysis of TTC staining of brain slices indicated a significant decrease in infarct size in sulindac treated animals at 4 mm, 6 mm, 8 mm and 10 mm from the anterior pole (Fig. 1) ($P<0.01$; 2 way ANOVA).

2.2. *Sulindac induces Hsp 27 expression in an ischemic model of stroke*

A previous study has indicated that sulindac induces Hsp 27 in heart as part of a late phase preconditioning mechanism. This was demonstrated using an *ex vivo* Langendorff myocardial ischemia model (Moench et al., 2009). Our current study indicates that sulindac treated animals express enhanced levels of Hsp 27 in penumbra and core of the ischemic area of the stroke model (left side) on day-3 (Fig. 2A) and day-11 (Fig. 2B) after vessel occlusion. Quantification of western blots by densitometry showed approximately a 2–3 fold increase in Hsp 27 expression on day-3 (Fig. 2A) and a 9 fold increase in Hsp 27 expression on day-11 (Fig. 2B) after sulindac treatment.

2.3. *Activation of Hsp 27 and Akt*

In several cell types Hsp 27 has been shown to modulate apoptosis by control of Akt activation. A previous investigation has identified Hsp 27 as an Akt substrate that dissociates from Akt upon phosphorylation (Rane et al., 2003). This study demonstrated that disruption of the interaction between Hsp 27 and Akt impairs Akt activation, leading to an enhanced rate of constitutive neutrophil apoptosis (Rane et al., 2003; Rane and Klein 2009). Furthermore, activation of Akt by Hsp 27 has been found to be necessary for cell survival. In the present study Akt is activated after ischemia. The sulindac treated groups show greater than 3-fold Akt activation in the penumbra of the ischemic model of stroke compared to the

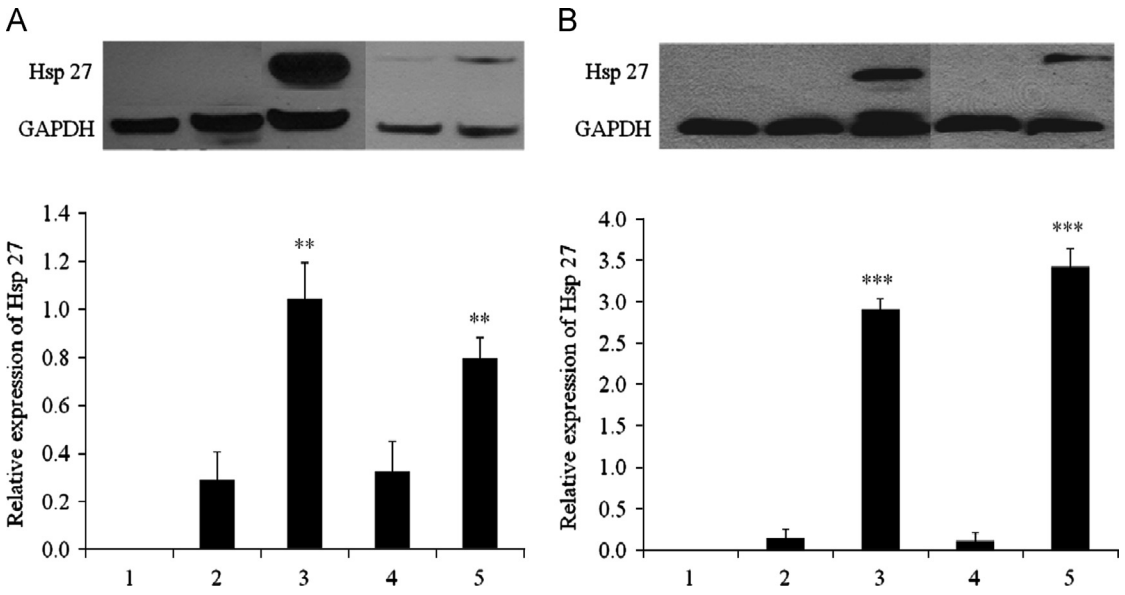

Fig. 2 – Sulindac increases Hsp 27 protein level in penumbra and core of ischemic area of stroke on day-3 (A) and day-11 (B). Results were from western blot analysis as described in Section 4. Mean results ($n=5$) of Hsp 27 protein level from densitometric scanning are presented. 1—Sham, 2—penumbra of control with no drug, 3—sulindac treated penumbra, 4—core of control with no drug, and 5—sulindac treated core (all data are presented as mean ± SEM, where $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

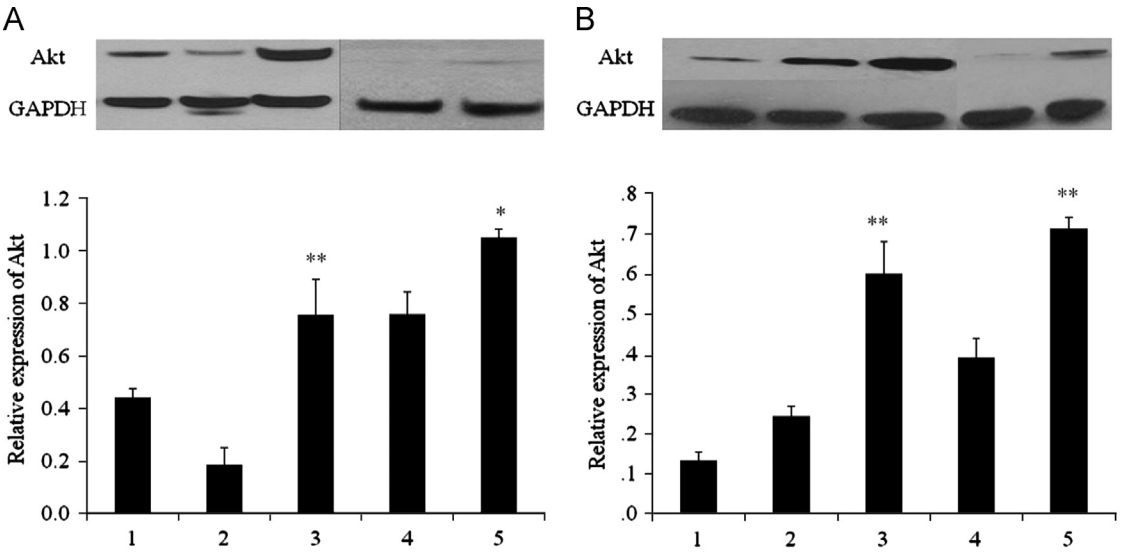

Fig. 3 – Akt protein level in penumbra and core of ischemic area of stroke on day-3 (A) and day -11 (B). Results were from western blot analysis as described in Section 4. Mean results ($n=5$) of Akt protein level from densitometric scanning are presented. 1—Sham, 2—penumbra of control with no drug, 3—sulindac treated penumbra, 4—core of control with no drug, and 5—sulindac treated core (all data are presented as mean ± SEM, where $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

penumbra of the untreated group and approximately a 50% increase in the core at day 3 after vessel occlusion (Fig. 3A). At day 11 after vessel occlusion the sulindac treated group shows a greater than 2-fold increase in Akt in the penumbra compared to the untreated group and an approximately 2-fold increase in the core compared to the untreated group (Fig. 3B). The sulindac treated groups show greater than 3-fold of phosphorylated Akt (P-Akt) in the penumbra and core of the ischemic model of stroke compared to the penumbra of the untreated group at day 3 after vessel occlusion (Fig. 4).

2.4. *Up-regulation of the anti-apoptotic protein Bcl-2 and down regulation of pro-apoptotic protein Bak*

The Bcl-2 families of proto-oncogenes encode specific proteins that regulate apoptosis induced by a variety of stimuli. Bcl-2 expression is induced in the brain by ischemia consistent with the concept that this protein could play a role as an endogenous neuro-protectant (Chen et al., 1997). This standpoint is supported by the observation that Bcl-2 protects neurons against ischemia when over-expressed either using

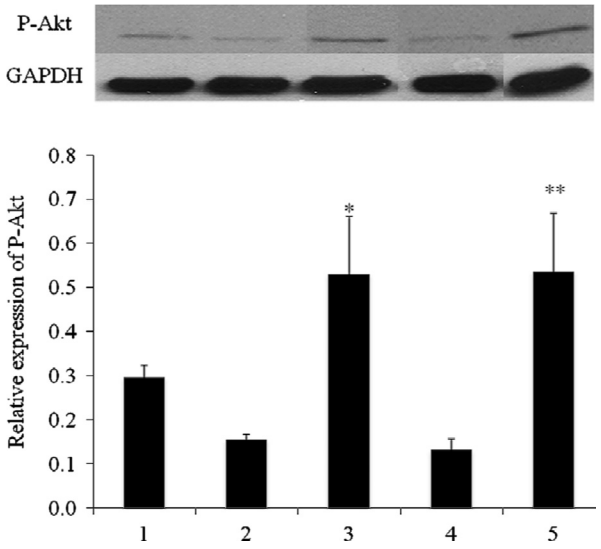

Fig. 4 – P-Akt protein level in penumbra and core of ischemic area of stroke on day-3. Results were from western blot analysis as described in Section 4. Mean results ($n=5$) of P-Akt protein level from densitometric scanning are presented. 1- Sham, 2—penumbra of control with no drug, 3—sulindac treated penumbra, 4—core of control with no drug, and 5—sulindac treated core (all data are presented as mean ± SEM, where $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

a viral vector or in transgenic animals (Linnik et al., 1995). Hence in this investigation we evaluated the effect of sulindac on expression of anti-apoptotic markers in relation to cell survival. Our analysis indicated that with sulindac treatment Bcl-2 expression increased at day 3 in the ischemic penumbra by approximately 2-fold relative to the untreated group (Fig. 5A). At day 11 after vessel occlusion the sulindac treated group showed a greater than 2-fold increase in Bcl-2 in the ischemic penumbra relative to the untreated group and showed a greater than 2.5-fold increase in Bcl-2 in the ischemic core relative to the untreated group (Fig. 5B).

Pro-apoptotic members are divided into the multi-BH domain "effectors" and the BH3- only proteins. The effectors comprise Bax and Bak. They share BH1-3 domains and have a transmembrane domain (Henshall and Engel, 2013). The Bcl-2 protein is known to promote cell survival while Bax, BAD, and/or Bak, all pro-apoptotic proteins, can translocate from the cytosol into the outer mitochondrial membrane after brain ischemia leads to apoptosis (Mohammad-Gharibani et al., 2014). In our study, the sulindac treated group showed a greater than 2.5-fold decrease in Bak in the ischemic penumbra relative to the untreated group and showed a greater than 2-fold decrease in Bak in the ischemic core relative to the untreated group on day-3 (Fig. 6A).

2.5. *Up-regulation of Bcl2/Bak and Bcl-2/PUMA ratio in ischemic penumbra and core*

Our result indicates that measurement of the ratio of pro-survival Bcl-2 to pro-death Bak on day 3 demonstrated a clear 3.5-fold up-regulation in penumbra and 1.5-fold up-regulation in core following sulindac treatment (Fig. 6C). PUMA, a p53-up-regulated modulator of apoptosis and a BH3-only member of the Bcl-2 protein family, is required for p53-dependent and independent forms of apoptosis. PUMA localizes to mitochondria and interacts with anti-apoptotic Bcl-2 and Bcl-XL or pro-apoptotic Bax/Bak in response to death stimuli (Steckley et al., 2007; Niizuma et al., 2009; Hong et al., 2010; Kuroki et al., 2009). PUMA has been shown to up-regulate Bax/Bak expression and induce neuronal apoptosis. Notably in our analysis while PUMA itself was not differentially regulated between conditions (Fig. 6B), measurement of the ratio of pro-survival Bcl-2 to pro-death PUMA (Bcl-2/PUMA) on day 3 demonstrated a clear 3.5-fold up-regulation following sulindac treatment in the ischemic penumbra (Fig. 6D).

2.6. *Sulindac up regulates GRP78 in ischemic core and penumbra*

Glucose-regulated protein 78 (GRP78) is the members of the heat shock protein family that act as the molecular chaperones in the folding and assembly of newly synthesized proteins within ER. Experimental evidences have indicated that up-regulation of GRP78 prevents neuronal damage induced by ER stress, and the increase in GRP78 expression may correlate to the degree of neuroprotection (Ye et al., 2013). Up regulation of GRP 78 leads to correction of protein mis-folding, and its protective roles are witnessed by the in vitro observations that GRP 78-depleted cells exhibited increased susceptibility to tunicamycin treatment and excitotoxicity (Morris et al., 1997; Yu et al., 1999; Xu et al., 2006). Our studies show that up-regulation of GRP 78 is specifically elicited in the sulindac treated ischemic penumbra on day 3 (Fig. 7A) and day 11(Fig. 7B) compared to the "no-drug" ischemic penumbra. Sulindac treated ischemic core and control ischemic core do not differ significantly in GRP 78 expression on day 3 but on day 11 we found a significant increase in GRP 78 expression in sulindac treated ischemic core compared to "no-drug" ischemic core (Fig. 7B).

3. Discussion

The aim of the current study was to investigate the neuroprotective role of sulindac in the rat MCAO model of focal cerebral ischemia. The studies presented here show that sulindac can protect neurons in the brain against oxidative damage resulting from ischemia and reperfusion. A key finding of the study demonstrated increased Hsp 27 expression following sulindac treatment. Several studies indicate that Hsp 27 has an anti-apoptotic role (Concannon et al., 2003) as well as a molecular chaperone role (Gabai and Sherman, 2002) in ischemia. Overexpression of Hsp 27 is also reported to decrease the amount of apoptosis-inducing factor (AIF), which is released by mitochondria in a caspase independent pathway of cell death (Stetler et al., 2008). Overexpression of wild-type heat shock protein 27 or a non-phosphorylable heat shock protein 27 mutant protects against ischemia/reperfusion injury in a transgenic mouse model (Hollander et al., 2004). Hsp 27 is also reported to protect the heart against myocardial infarction (Efthymiou

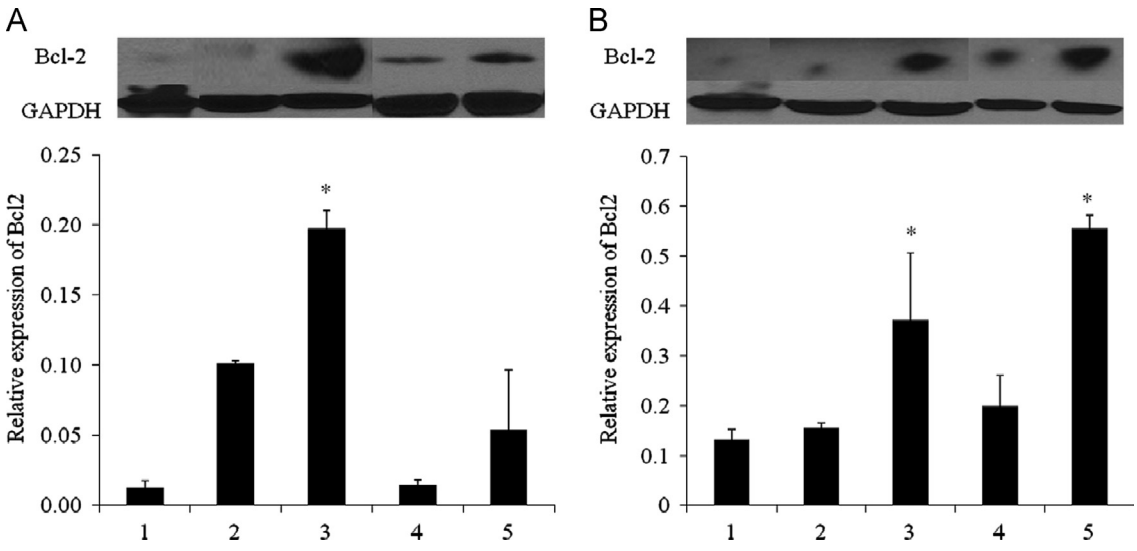

Fig. 5 – Bcl-2 protein level in penumbra and core of ischemic area of stroke on day-3 (A) and day-11 (B). Results were from western blot analysis as described in Section 4. Mean results ($n=5$) of Bcl-2 protein level from densitometric scanning are presented. 1—Sham, 2—penumbra of control with no drug, 3—sulindac treated penumbra, 4—core of control with no drug, and 5—sulindac treated core. (All data are presented as mean ± SEM, where *$P<0.05$, **$P<0.01$, ***$P<0.001$).

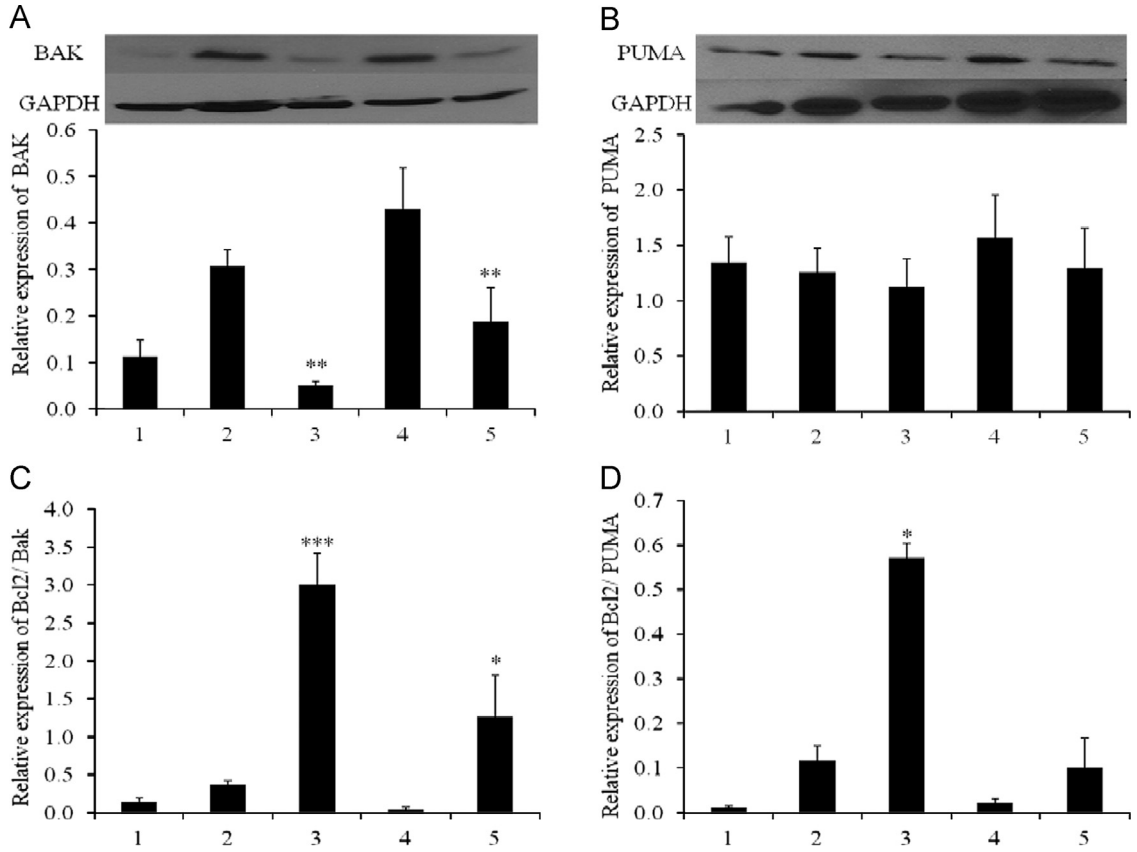

Fig. 6 – Bak (A) and PUMA (B) protein level in penumbra and core of ischemic area of stroke on day-3. Bcl2/Bak ratio expression (C) and Bcl2/PUMA ratio expression (D) are in penumbra and core of ischemic area of stroke on day-3. Results were from western blot analysis as described in Section 4. Mean results ($n=5$) of Bak and PUMA protein level from densitometric scanning are presented. 1—Sham, 2—penumbra of control with no drug, 3—sulindac treated penumbra, 4—core of control with no drug, and 5—sulindac treated core (all data are presented as mean ± SEM, where *$P<0.05$, **$P<0.01$, ***$P<0.001$).

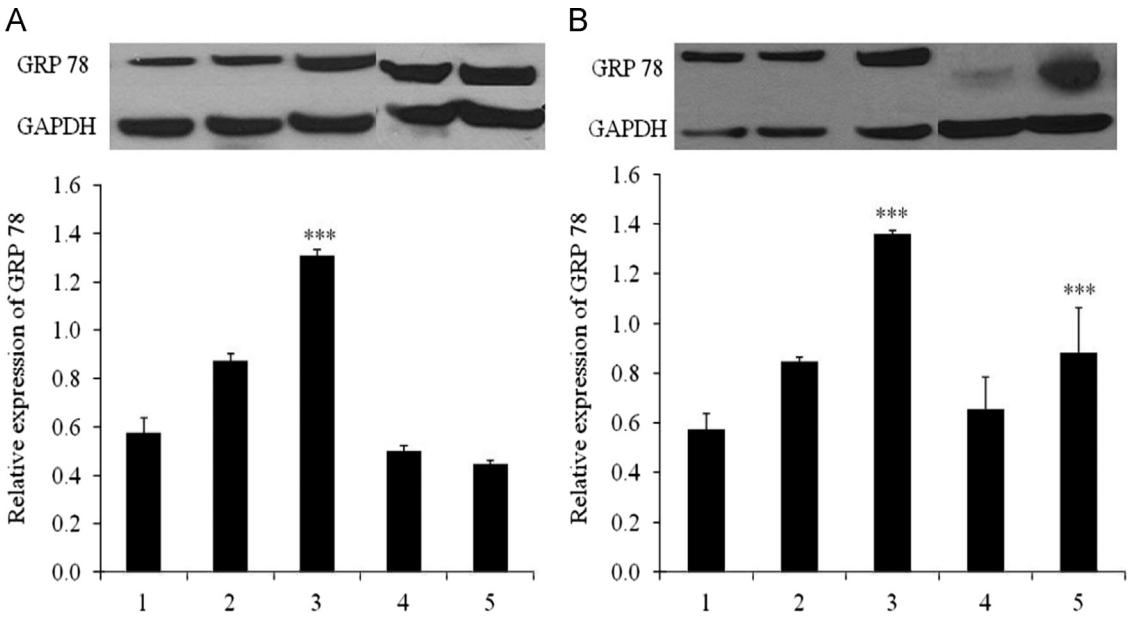

Fig. 7 – GRP 78 protein level in penumbra and core of ischemic area of stroke on day-3 (A) and day-11 (B). Results were from western blot analysis as described in Section 4. Mean results ($n=5$) of GRP 78 protein level from densitometric scanning are presented. 1—Sham, 2—penumbra of control with no drug, 3—Sulindac treated penumbra, 4—core of control with no drug, and 5—sulindac treated core (all data are presented as mean ± SEM, where $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

et al., 2004). Our study on MCAO in rats indicates that sulindac induces Hsp 27 expression on days 3 and 11. There are certain key studies in the literature suggesting that sulindac can protect cells against free radical damage. However to date no previous studies show a prolonged protective effect of sulindac in an in vivo model of cerebral ischemia.

Hsp 27 has been shown to protect cells in vitro by interfering with stress-triggered apoptotic cascades (Badin et al., 2006). Both Akt and Hsp 27 are reported to promote cell survival by inhibiting apoptosis. Introduction of anti-Hsp 27 antibodies into neutrophils was found to block Hsp 27's interaction with Akt thus inhibiting Akt activation indicating that Hsp 27 association with the Akt signaling complex is necessary for Akt activation in these cells (Rane et al., 2003). Hsp 27 has been identified in several systems as a "scaffold" protein, capable of leading to the activation of pro-survival signaling molecules such as MK2 and Akt (Stetler et al., 2009). If the Akt–Hsp 27 interaction is intact, then upon stimulation of cells with a ligand, Akt translocates to the membrane and is fully activated by phosphorylation on Thr308 and Ser473 by PDK1 and PDK2 respectively (Rane and Klein 2009).

Sulindac treated penumbra showed greater Bcl-2 expression and lower Bak/PUMA expression compared to the control ischemic penumbra. Bcl-2 is known to play a central role in regulating neuronal survival (Davies, 1995). Bcl-2-associated X protein (Bax) is also required for oxidative stress induced cell death and PUMA plays a dominant role in regulating Bax/Bak activation and neuronal apoptosis (Steckley et al., 2007). Hence sulindac exerts its neuroprotective role by inhibiting apoptosis via increased Bcl-2 expression and decreased Bak/PUMA expression in the ischemic penumbra.

GRP 78 is an endoplasmic reticulum (ER)-resident molecular chaperone whose expression serves as a good marker of ER stress (Ma and Hendershot, 2001). Although the ER is implicated in neuronal degeneration in some situations, its role in delayed neuronal cell death after ischemia remains uncertain. However in previous studies it was shown that ER stress is reduced by ischemic preconditioning and that ER stress reduction by preconditioning was the result of ER molecular chaperone induction (Hayashi et al., 2003).

When severe ischemia is induced increased levels of unfolded proteins occur in the ER lumen and this is associated with increases in ER chaperones such as GRP78. In the present study up-regulation of GRP78 in the sulindac treated core and penumbra at day 3 and day 11 may contribute to a protective response in the same way as was reported in previous reports on preconditioning against ER stress in delayed neuronal death (Davies, 1995).

In summary, Hsp 27 controls apoptosis by regulating Akt activation (Rane et al., 2003). Numerous studies have demonstrated that up-regulation of Hsp27 is protective for neurons under ischemic conditions. Therefore sulindac may act therapeutically in cerebral ischemia through its effects on Hsp 27 and Akt. As Hsp 27 and Akt prevent apoptosis it is likely that the elevated levels of the anti-apoptotic molecule Bcl-2 in the current study will further augment the pro-survival effect of sulindac. An enhanced expression of Grp78 in the sulindac treated ischemic core and ischemic penumbra implies that sulindac protects through preconditioning and is consistent with previous studies on ischemic preconditioning showing an enhanced level of GRP78 expression and protection against slow neuronal death.

Although other NSAIDs may show protection in focal brain ischemia, some of these may act primarily by anti-inflammatory actions, whereas sulindac has been shown to function in several capacities: as a catalytic antioxidant, or an anti-inflammatory agent or as a preconditioning agent (Moench et al., 2009; Etienne et al., 2003). Previous studies on

the NSAID, parecoxib (Ye et al., 2013) indicate that it protects against apoptosis in a rat MCAO model via GRP78 activation similarly to what we observed for sulindac. Interestingly parecoxib decreased CHOP activity providing evidence that the drug decreased ER stress responses. The NSAID flurbiprofen (Sun et al., 2011) was recently shown to protect against focal ischemia at 24 h post-occlusion through up-regulation of pro-survival BCl-2 family members and through increased p-Akt expression. In our own investigation increased p-Akt levels point to a pro-survival action concurrent with Hsp27 activation. Such increases in Akt and p-Akt are known to be anti-apoptotic and may also be consistent with the action of sulindac occurring through established preconditioning mechanisms (Prasad et al., 2011).

The protective effect we have observed with sulindac in cerebral ischemia is contrary to several studies that NSAIDs cause increased risk of stroke and heart attack (Hennekens and Borzak, 2008). It should be noted that in the rat feeding experiments described in this study the daily dose of sulindac (0.2 mg/day) was only 10–15%, on a weight basis, compared to the doses taken clinically as an anti-inflammatory agent. Hence sulindac at the low dose administered in this study is likely to be very valuable as a neuro-protective agent against oxidative stress in cerebral ischemia.

4. Experimental Procedure

4.1. *Experimental design for day-3 and day-11:*

Group 1: sham animals ($n=5$),
Group 2: "no-drug" ischemic untreated animals (MCAO) ($n=5$),
Group 3: treated animals with MCAO (pre and post-sulindac inj.) ($n=5$).

4.2. *Preparation for surgery and establishment of transient model of stroke*

Male adult Sprague-Dawley rats (weighing 250–300 g) were subjected to middle cerebral artery occlusion (MCAO) according to standard procedures. Rats were anaesthetized initially with Isoflurane and then during surgery with Ketamine 80 mg/kg and xylazine 20 mg/kg (intraperitoneal injection) and were allowed spontaneous respiration throughout surgical procedure. The body temperature was maintained at 37.5 °C using a heating pad connected to rectal probe (CMA 450) throughout surgery. After attaining deep anesthesia, hair was removed and the surgical area prepared aseptically. The Laser Doppler flow (LDF) (Perimed Inc, OH, USA) was positioned on the skull above the cerebral cortex of the left hemisphere in the supply territory of the MCA. After attaching the Doppler probe, rats were gently turned upside down to create the MCAO model in the supine position. Rat cerebral blood flow (CBF) was monitored continuously with a 1.0 s time constant from before the surgery for MCAO. A ventral midline cervical incision was made. Under a surgical scope, the left common carotid artery (CCA) along with external carotid artery (ECA) and internal carotid artery (ICA) were exposed via a midline pretracheal incision. The vagus and sympathetic nerves were separated carefully from the artery. The external carotid artery (ECA) was ligated 2 mm distal to the bifurcation of the common carotid artery. The internal carotid artery was dissected distally to expose the origin of the pterygopalatine artery (PPA). The common carotid artery was then ligated temporarily 5–10 mm proximal to its bifurcation and the pterygopalatine artery was ligated close to its origin with a 5-0 nylon suture (Chen et al., 2008). The micro-incision was made on ECA and monofilament suture uncoated nylon 4.0 inserted through ECA to ICA. The filament was advanced 18–22 mm from the carotid artery bifurcation into the internal carotid artery until there was slight resistance (O'Neill and Clemens, 2001). Reperfusion was accomplished by withdrawing the filament 2 h after MCAO (Popp et al., 2009). CBF measured during surgery until 30 min after reperfusion. The surgical wound was closed with 4-0 silk suture. In sham-operated rats, rectal temperature and Laser doppler flow (LDF) was monitored continuously for 2 h after placement of the intraluminal suture in the ECA, without advancing it to the point of causing ischemia (2 mm past the carotid bifurcation). ($n=5$ per group) (Srinivasan and Sharma, 2011).

4.3. *Preparation and dosage of sulindac drug*

50 mg sulindac powder (sigma) is dissolved in 1 ml of 500 mM Tris (pH 8) solution. The drug was given via subcutaneous injection (0.2 mg/day) 2 days pre-surgery (Moench et al., 2009) and every 24 h post surgery until sacrifice on day 3 or 11.

4.4. *2,3,5-Triphenyltetrazolium chloride (TTC) assessment of lesion size*

Rats were euthanized 3 or 11 days after surgery. The brain was quickly removed and sectioned into 2 mm thick slices starting at the frontal pole using a Brain Matrix Slicer (Zivic instruments, PA, USA). Slices were immersed in 2% TTC (J.C. Baker, India) in a Petri dish and incubated at 37 °C for 5 min. TTC, a water soluble salt, is reduced by mitochondrial dehydrogenases to formazan, a red, lipid-soluble compound that turns normal tissue deep red (Bederson et al. 1986; Rich et al., 2001). Thus, reduced TTC staining identifies regions of diminished mitochondrial function in the ischemic tissue (Li et al., 1997). To assess lesion volume, TTC-stained slices (2, 4, 6, 8, 10, and 12 mm from the frontal pole) were scanned and analyzed by Image-J analysis software (public domain software: URL: http://rsb.info.nih.gov/nihimage/). Lesion volume was determined as the percent of the total ipsilateral hemispheric volume and was calculated as:

$$[(VC-VL)/VC]100$$

where VC is the volume of the contralateral hemisphere and VL is the volume of non-lesioned tissue in the lesioned hemisphere (Mohammad-Gharibani et al., 2014; Gharibani et al., 2013). Then, these sections were compared in different treated and untreated animal groups.

4.5. *Sample collection for western blot analysis*

After TTC experiment, while the sections were on ice, the lesioned part of the left hemisphere (Core and Penumbra) (Gharibani et al., 2013) and the right hemisphere (identical parts) were quickly dissected on dry ice and homogenized in Lysis buffer consisting of 50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, pH 8.0, 1% Triton-X-100, 1:100 dilution of mammalian protease inhibitors (Sigma-Aldrich, MO, USA) and protease inhibitor (Buddhala et al., 2012) for immunoblotting. Protein concentrations of each sample solution were determined with a Bradford protein assay, and samples were stored at −80 °C until use. Protein samples were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk for 1 h and then probed overnight with Hsp 27, Hsp 70, GRP 78, Bcl-2, PUMA and Akt(1:1000) antibodies, washed 3 × 20 min in TBS-T containing 0.1% Tween 20, and incubated with horseradish peroxidase-conjugated anti-Rabbit IgG (1:3000) for 90 min. Bound antibody was visualized with the ECL system (Amersham) (Buddhala et al., 2012; Mohammad-Gharibani et al., 2013). Membranes were then stripped and probed for GAPDH to confirm equal protein loading. Size and density of the bands were measured by Image-J analysis software, and signals were quantified relative to the signal intensity for GAPDH.

4.6. *Data and statistical analysis*

All results are presented as means ± SE. Statistical analysis was performed with the use of XL stat (Microsoft) and SPSS (IBM) Statistical software. ANOVA and Student's *t*-test were used to determine statistical significance. Statistic significance was set at $P < 0.05$

Disclosure/conflict of interest

There is no conflict of Interest

Acknowledgments

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