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*Original Article*

# ASIC1a contributes to neuroprotection elicited by ischemic preconditioning and postconditioning

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**Abstract:** Acid-sensing ion channels, ASICs, are proton-gated cation channels widely expressed in peripheral sensory neurons and in neurons of the central nervous system that play an important role in a variety of physiological and pathological processes. To further confirm the role played by ASIC1a in cerebral ischemia, here we examined the involvement of this channel in two endogenous recently characterized neuroprotective strategies: brain ischemic preconditioning and postconditioning. The main aim of this study was to elucidate whether ASIC1a might take part as effector in the neuroprotection evoked by brain ischemic preconditioning and postconditioning. For this purpose we investigated the effect of ischemic preconditioning and postconditioning on (1) ASIC1a mRNA and protein expression in the temporoparietal cortex of rats at different time intervals; and (2) the effect of p-AKT inhibition on ASIC1a expression during ischemic preconditioning and postconditioning. Ischemic preconditioning and postconditioning were experimentally induced in adult male rats by subjecting them to different protocols of middle cerebral artery occlusion and reperfusion. ASIC1a expression was dramatically reduced in both the neuroprotective processes. These changes in ASIC expression were p-AKT mediated, since LY-294002, a specific p-AKT inhibitor, was able to prevent variations in ASIC1a expression. The results of the present study support the idea that the downregulation of ASIC1a expression and activity might be a reasonable strategy to reduce the infarct extension after stroke.

**Keywords:** ASIC1a, preconditioning, postconditioning, stroke, neuroprotection

## Introduction

Acid-sensing ion channels, ASICs, are proton-gated cation channels widely expressed in peripheral sensory neurons and in neurons of the central nervous system. Recent studies have demonstrated that the activation of these channels plays an important role in a variety of physiological and pathological processes such as nociception, mechanosensation, synaptic plasticity, and acidosis-mediated neuronal injury. In fact, a significant drop of tissue pH or acidosis is a common feature of acute neurological conditions such as ischemic stroke, brain trauma, and epileptic seizures. In particular, several studies have shown that ASIC1a blockade or genetic ablation is able to induce a strong, long lasting protection in animal models of cerebral ischemia, thus suggesting that ASIC1a can be considered an important drug-gable target in the process of identifying an effective stroke therapy [1, 2].

To further confirm the role played by ASIC1a in cerebral ischemia, we examined involvement of this channel in two recently characterized endogenous neuroprotective strategies: brain ischemic preconditioning and postconditioning.

It has been recently reported that ischemic preconditioning, a sub-lethal ischemic episode applied before a longer harmful ischemia [3-5], and ischemic postconditioning, a sub-lethal ischemia subsequent to a prolonged harmful ischemic episode [6-16], are both able to exert a remarkable neuroprotection. Therefore, the molecular characterization of ischemic preconditioning and postconditioning is of fundamental importance to establish the neuroprotective mechanisms elicited by these two strategies.

The molecular mechanisms contributing to the tissue-protection mediated by preconditioning and postconditioning have been classified as: (1) triggers, like adenosine (ADO), opioids, erythropoietin (EPO), nitric-oxide, reactive oxygen species, cytokines and bradykinin; (2) mediators, like reperfusion injury salvage kinase (RISK) pathways and other protein kinases; and finally (3) effectors, like mitochondrial permeability transition pore and mitochondrial potassium ATP channels [15, 17, 18]. Many molecular determinants have been suggested as putative effectors for this clinically relevant neuroprotective condition. In particular, the well known family of Mitogen Activated Protein Kinases (MAPK) has been proposed as an important mediator of these two neuroprotective conditions [11, 19].

The main aim of this study was to elucidate whether ASIC1a might take part as effector in the neuroprotection evoked by brain ischemic preconditioning and postconditioning. For this purpose we investigated the effect of ischemic preconditioning and postconditioning on (1) ASIC1a mRNA and protein expression in the temporoparietal cortex of rats at different time intervals; and (2) the effect of p-AKT inhibition on ASIC1a expression during ischemic preconditioning and postconditioning.

## Methods

*Experimental groups*

Male Sprague–Dawley rats (Charles River) weighting 250 to 300 g were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of "Federico II", University of Naples, Italy.

*Focal ischemia*

Transient focal ischemia was induced, as previously described [11], by suture occlusion of the middle cerebral artery (MCA) in male rats anesthetized using 1.5 % sevofluorane, 70% $N_2O$, and 28.5% $O_2$. Ischemia was induced by introducing a 3-0 surgical monofilament nylon suture (Doccol, CA, USA) from the external carotid artery into the internal carotid artery and advancing it into the circle of Willis to the branching point of the MCA, thereby occluding the MCA [20]. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the right MCA. Cerebral blood flow was monitored through a disposable microtip fiber optic probe (diameter 0.5 mm) connected through a Master Probe to a laser Doppler computerized main unit (PF5001; Perimed, Sweden) and analyzed using PSW Perisoft 2.5 [11]. Animals that did not show a cerebral blood flow reduction of at least 70% were excluded from the experimental group, as well as animals that died after ischemia induction. Rectal temperature was maintained at 37±0.5°C with a thermostatically controlled heating pad and lamp. All surgical procedures were performed under an operating stereomicroscope.

*Preconditioning experimental protocol*

Ischemic preconditioning was induced as previously described [11]. Briefly, 72 hours before 100 min of MCAO, a transient occlusion of the MCA was induced for 30 min. Animals were then recovered for 24 hours. The success of the experimental procedures was confirmed by measuring CBF in all the experimental steps.

*Postconditioning experimental protocol*

Ischemic postconditioning was induced as previously described [11]. Briefly, after 100 min of MCAO, reperfusion was established for 10 min after which the MCA was re-occluded for 10 min. Animals were then recovered for 24 h. The success of the experimental procedures was confirmed by measuring CBF in all the experimental steps.

*Use of p-AKT inhibitor*

To assess the effect of p-AKT on ASIC1a expression, 10 rats were treated 15 minutes before preconditioning or postconditioning induction with the inhibitor of phosphatidylinositol-3 kinase LY-294002 (5 μL, 10 μmol/L, in 3% dimethyl sulfoxide) or vehicle alone (3% dimethyl sulfoxide).

LY-294002 was intracerebroventricularly injected at the following coordinates from the bregma: anteroposterior, -0.4; laterolateral, -2.0; depth, -2.5 [21].

*Western blotting analysis*

Cortical samples were harvested from ischemic brains of rats subjected to 100 minutes of

MCAO or from brains of pre- or post-conditioned rats. In all the experimental conditions, two groups of ipsilateral and contralateral temporoparietal cortex were obtained at different reperfusion times after the last occlusion: (a) 0.5 hours; (b) 5 hours; and (c) 24 hours. Two groups of samples were obtained from brains of sham-operated animals.

Rat brain samples were homogenized using an 18-gauge needle in a lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1% Triton X-100, 0.2 mmol/L Sodium orthovanadate) containing protease inhibitor cocktail (aprotinin, leupeptin, and pepstatin) (Roche Diagnostic, Monza, Italy). After centrifugation at 13,400 rpm at 4°C for 20 minutes, the supernatants were collected. Protein concentration was estimated using the Bradford reagent (Bio-Rad Laboratories, Segrate, Milan, Italy). Then, 100 µg of protein was mixed with a Laemmli sample buffer. The samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto Amersham™ Hybond™-ECL nitrocellulose membranes (GE Healthcare, Milan, Italy). The non-specific binding sites were blocked with an incubation of 5% non-fat dry milk in Tris-buffered saline (TBS) 0,1% Tween 20 (Sigma-Aldrich, Milan, Italy) for 2 hours at room temperature. The blots were then probed with polyclonal anti-ASIC1 (1:1000, Alpha Diagnostic, San Antonio, TX, USA) and monoclonal anti-β-Actin (1:1000, Sigma-Aldrich, Milan, Italy) diluted in Tris-buffered saline 5% non-fat milk overnight (4°C) and detected using horseradish peroxidase-conjugated secondary antibody (1:2000, GE Healthcare, Milan, Italy) for 1 hour at room temperature in 5% non-fat dry milk. Immunoreactive bands were detected with the ECL reagent (GE Healthcare, Milan, Italy). The optical density of the bands was determined by Chemi-Doc Imaging System (Bio-Rad Laboratories, Segrate, Milan, Italy).

*mRNA expression analysis by Real-Time PCR*

Rat cortex dissected from the ipsilesional hemisphere of sham-operated and postconditioned rats 5 and 24 hours after surgery were frozen on dry ice. Brain samples were ground into powered dry ice, then Trizol Reagent solution (Invitrogen, Italy) was added. Total RNA was extracted and purified in accordance with the manufacturer's protocol. For reverse transcription, 2.0 µg of each extracted RNA was digested with DNase and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Canada). Amplification was performed using Power Sybr Green PCR Master Mix (Applied Biosystem, Milan, Italy) according to the manufacturer's protocol. All data were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA levels and expressed as percentage of the mRNA levels detected in sham-operated animals. Sequences of the primers used were the following: HPRT: F (444-467) TGGAAAGAACGTC TTGATTGTTGA; R (528-507) GCTGTACTGCTTGAC CAAGGAA; ASIC1a: F (1581-1598) GGAAGGGAG CCACGATGA; R (1645-1624) TGATGGTTTCAGAG TGGTTTGG.

*Statistic analysis*

Values are expressed as means ± S.E.M. Statistical analysis was performed with 2-Way ANOVA, followed by Newman-Keuel's test. Statistical significance was accepted at the 95% confidence level ($p < 0.05$).

## Results

*Both ischemic preconditioning and postconditioning induce ASIC1a downregulation in the peri-ischemic temporoparietal cortex*

In order to assess the role played by ASIC1a in ischemic preconditioning and postconditioning, its expression was evaluated in the ipsilesional temporoparietal cortex of rats subjected to ischemic pre- or post-conditioning at different reperfusion time intervals: 0.5 h, 5 h, and 24 hours, and compared to the expression of ASIC1a in the same brain region after tMCAO alone (**Figure 1**). ASIC1a showed a dramatic change in its expression. After preconditioning alone (**Figure 1B**), preconditioning plus ischemia (**Figure 1C**), and ischemic postconditioning (**Figure 1D**), a dramatic reduction of more than 50% in ASIC1a expression was observed at all the considered time-intervals. Interestingly, after harmful ischemia the expression of ASIC1a did not change (**Figure 1A**).

*ASIC1a mRNA expression is downregulated by ischemic preconditioning and postconditioning*

To assess whether ASIC1a protein downregulation was accompanied by a similar behave of the mRNA, Real-Time PCR was performed in the ipsilesional temporoparietal cortex of rats sub-

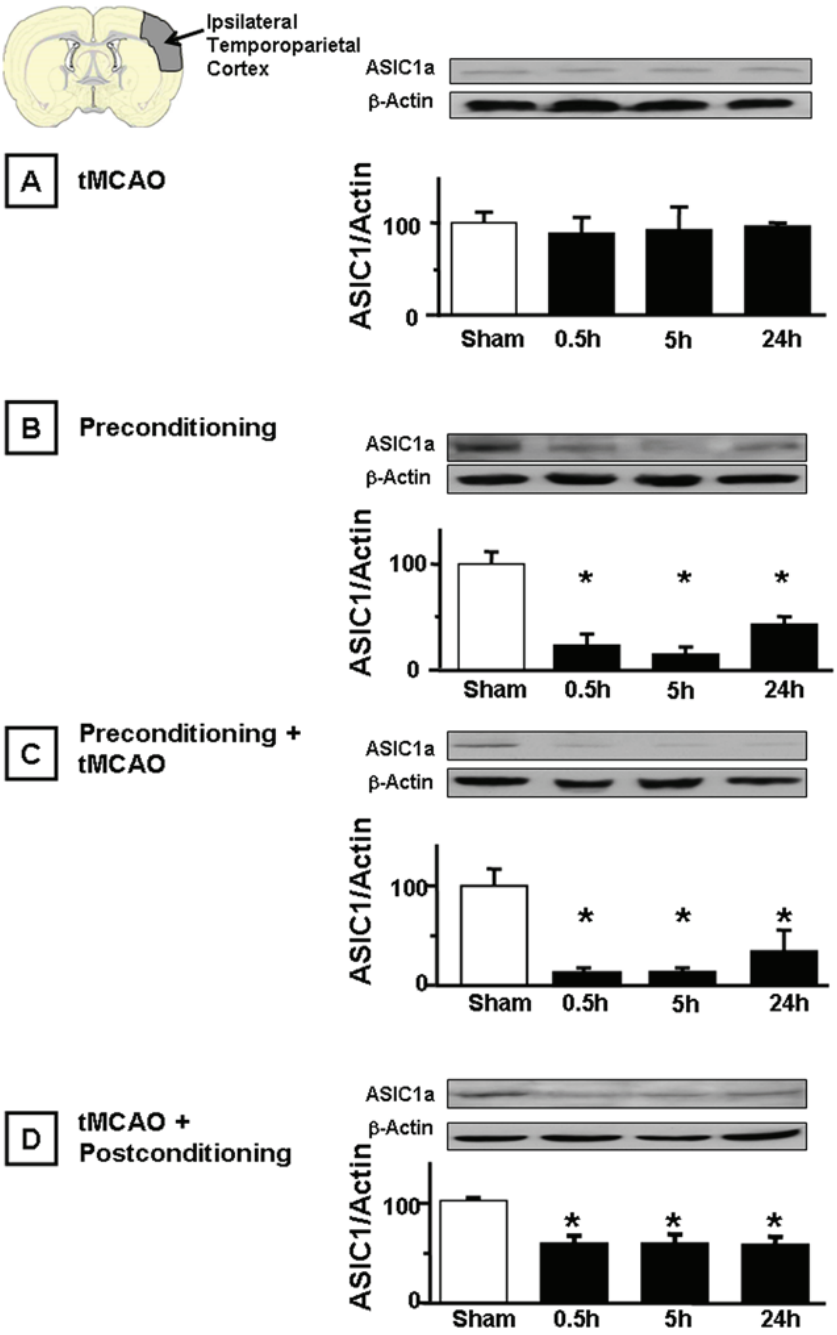


**Figure 1.** ASIC1a protein expression in rat ipsilateral cortex after ischemia, preconditioning and postconditioning. Time course of ASIC1a protein (70kDa) expression after tMCAO (A), preconditioning (B), preconditioning plus tMCAO (C) and tMCAo + postconditioning (D) in the ipsilateral temporoparietal cortex. A representative brain slice cartoon indicating the area of interest is on the top of the figure. Data were normalized on the basis of β-actin levels and expressed as percentage of sham-operated controls (Sham). On the x-axis is the reperfusion time interval. Values are mean± SEM. *p<0.05, compared with Sham. n=6-8 animals for each column.

jected to the same experimental conditions at different reperfusion time intervals: 0.5 h, 5 h, and 24 hours, and compared to the expression of the ASIC1a mRNA in the same brain region of sham-operated animals. Results showed that, after preconditioning alone, ASIC1a mRNA was

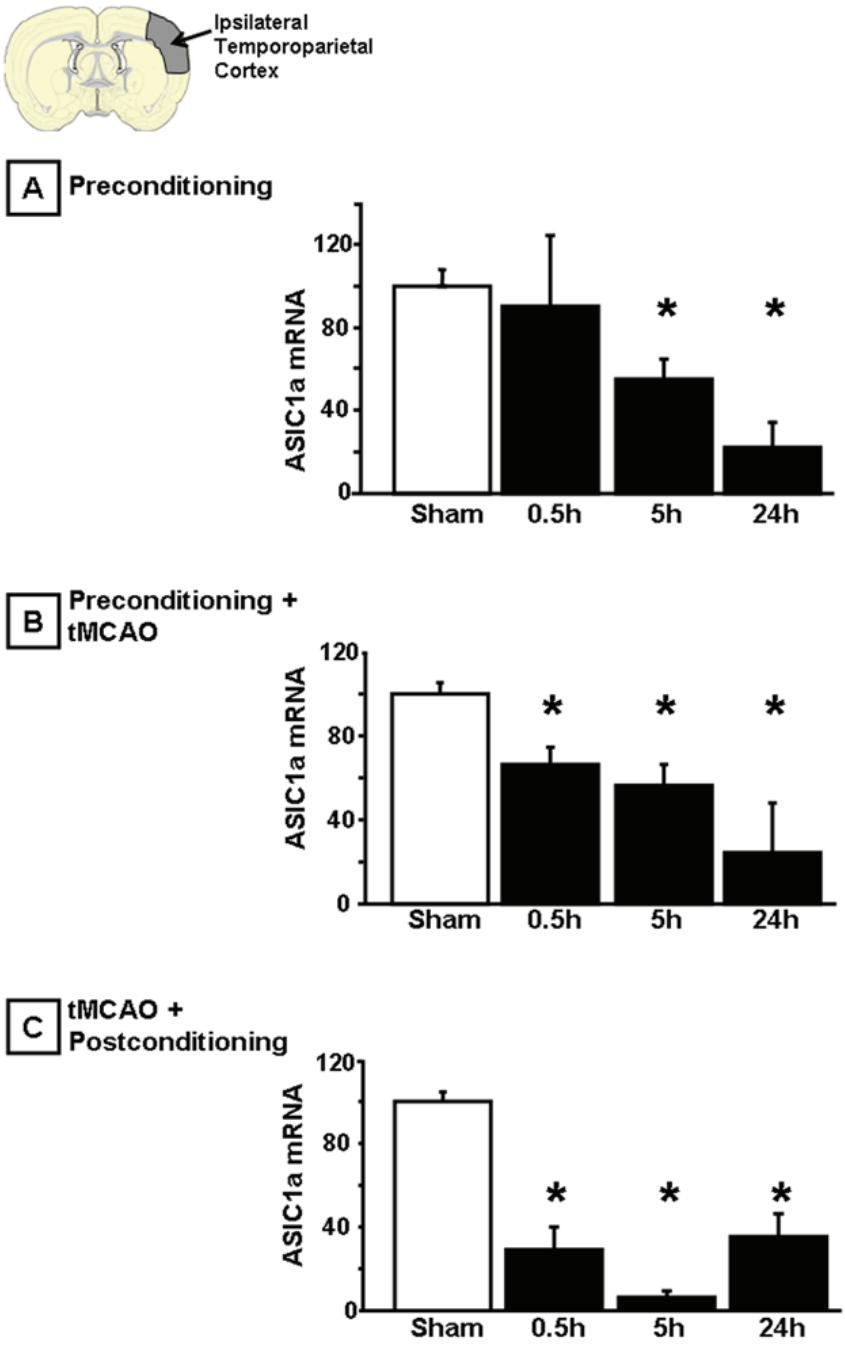


**Figure 2.** ASIC1a mrna expression in rat ipsilateral cortex after ischemic preconditioning and postconditioning. Time course of ASIC1a mRNA expression after preconditioning alone (A), preconditioning plus tMCAO (B) and tMCAO + postconditioning in the ipsilateral temporoparietal cortex. A representative brain slice cartoon indicating the area of interest is on the top of the figure. On the x-axis is the reperfusion time interval. Data were normalized on the basis of GAPDH levels and expressed as percentage of sham-operated controls (Sham). Values are mean± SEM. *p<0.05, compared with Sham. n=6-8 animals for each column.

reduced by almost 50% starting at 5 h of reperfusion (**Figure 2A**). Similarly, after preconditioning followed by 100 min tMCAO (**Figure 2B**) or after 100 min tMCAo followed by postconditioning (**Figure 2C**), the ASIC1a mRNA reduction was evident at earlier time point, being already evident at 0.5 h (almost 30%). This reduction was still present 5 h and 24 hours later (**Figure**

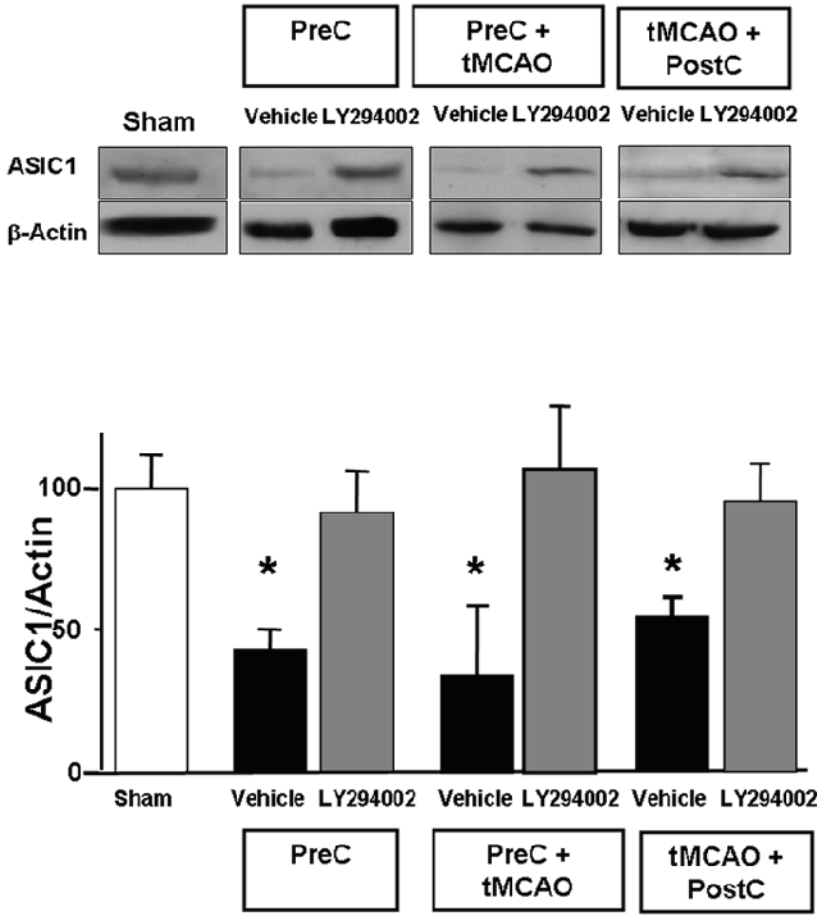


**Figure 3.** Effect of the p-akt inhibitor ly2940002 on asic1a downregulation induced by preconditioning and postconditioning treatments. Representative Western blots of ASIC1a protein levels in the temporoparietal cortex of sham-operated animals (Sham), rats subjected to preconditioning alone, preconditioning plus tMCAO or tMCAO + postconditioning icv administered with the vehicle, and rats subjected to the same experimental conditions icv administered with the p-AKT inhibitor LY294002. Values are mean ± SEM. *p<0.05, compared with control group. n=6-8 animals for each column.

2B and 2C).

*The p-AKT Inhibitor LY294002 reverts ASIC1a downregulation induced by ischemic preconditioning and postconditioning*

As it has previously shown that the neuroprotective effect induced by ischemic preconditioning and postconditioning is in part mediated by p-AKT activation [11,12], we determined whether ASIC1a downregulation induced by ischemic pre - and post-conditioning was also p-AKT mediated. The p-AKT inhibitor LY294002 was intracerebroventriculalrly (icv) injected before pre- or post-conditioning induction [22] and ASIC1a expression was evaluated in the ipsilesional temporoparietal cortex of rats subjected to the three experimental conditions at 24 hours of reperfusion and compared to the expression of the protein in the same brain region after ischemia alone. The results obtained showed that the reduction in ASIC1a protein expression induced by preconditioning alone, preconditioning plus tMCAO and tMCAO plus post-conditioning was reverted by the p-AKT inhibitor LY294002 (**Figure 3**), thus suggesting that the ASIC1a downregulation induced by pre- and post-conditioning is mediated through p-AKT activation.

## Discussion

In this study we demonstrated that the brain isoform ASIC1a represents an additional new

molecular effector involved in the neuroprotection elicited by ischemic preconditioning and postconditioning. In particular, we demonstrated that p-AKT is the mediator of this action since the selective p-AKT inhibition prevented ASIC1a downregulation induced by both preconditioning and postconditioning thus reverting the conditioning-induced neuroprotection.

From the results obtained in the present study it is possible to state that preconditioning and postconditioning may exert neuroprotective effect through p-AKT-dependent downregulation of ASIC1a. P-AKT in turn is up-regulated by ischemic preconditioning and postconditioning. The results showing that p-AKT is the mediator of the downregulation of ASIC1a during pre- and post-conditioning are supported by previous findings showing that both neuroprotective strategies in the brain involve the activation of the family of protein kinases called Reperfusion Injury Salvage Kinases, RISK, including Akt [11, 12, 23]. In fact, during harmful ischemia, Akt is transiently phosphorylated, and consequently activated, only for a short interval of time after reperfusion [11], whereas after pre- and post-conditioning, the phosphorylation of Akt persists longer, being still present in the phosphorylated form even 24 h later [11], the same time interval at which an ASIC1a downregulation occurs.

The neuroprotection induced by ASIC1a downregulation is in line with previous results showing that ASIC1a gene ablation or ASIC1a blockade induce a dramatic neuroprotection.

Summarizing the results so far obtained in studies devoted to understand the role of ASICs in the stroke pathophysiology, it is possible to state that acidosis occurring in cerebral ischemia is able to activate ASICs in in vivo and in vitro models of stroke. In particular, studied conducted in cortical neurons subjected to conditions mimicking cerebral ischemia, showed that, when studied at resting membrane potential, acidosis activated a depolarizing current mediated by influx of cations; this required activation of ASIC1a in particular, because this response was abolished in neurons cultured from ASIC1a−/− mice [2]. If neurons cultured from wild-type mice were deprived of oxygen and glucose, thereby mimicking some features of ischemia, acidosis resulted in a larger and more sustained current. Interestingly, $Ca^{2+}$ permeated the acidosis-activated channel and the influx of $Ca^{2+}$ contributed to acidosis-induced death of neurons in vitro. Most importantly, limiting activation of ASICs reduced the damage produced by ischemia in vivo by approximately 60%, a conclusion based on both pharmacological evidence and study of ASIC1a−/− mice. Furthermore, the pharmacologic and genetic interventions limiting ASIC1 activation exerted neuroprotective effects even in the presence of a glutamate receptor antagonist [2].

Multiple molecular targets have been identified for which neuroprotective therapies have been shown to be effective in animal models including nitric oxide synthase, cannabinoid receptors, and many others. What sets apart the results obtained with ASICs is the simple yet plausible mechanism: the marked drop of pH damages the ischemic tissue and does so by activation of this ASIC receptor. Importantly, the drop of pH in the core of the ischemic lesion raises the possibility that preventing activation of the ASIC receptor may reduce damage in the core, not simply in the partially ischemic tissue surrounding the core that is targeted by most neuroprotective treatments. Thus, the results provided in the present study support the hypothesis that ASIC1a blockade or downregulation is protective and the discoveries made in this field of research raise the exciting possibility that small molecules could be developed to prevent acidosis-mediated activation of this receptor and thereby reduce ischemic injury.

Altogether, our data support the importance of p -AKT in mediating preconditioning and postconditioning neuroprotection and suggest ASIC1a as one of the additional signals downstream to p-AKT and involved in the neuroprotective effect of ischemic pre- and post-conditioning.

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