Original Contribution

ORAI1/STIM1 Interaction Intervenes in Stroke and in Neuroprotection Induced by Ischemic Preconditioning Through Store-Operated Calcium Entry

Agnese Secondo, PhD*; Tiziana Petrozziello, PhD*; Valentina Tedeschi, PhD; Francesca Boscia, PhD; Antonio Vinciguerra, PhD; Roselia Ciccone, PhD; Anna Pannaccione, PhD; Pasquale Molinaro, PhD; Giuseppe Pignataro, PhD, MD; Lucio Annunziato, MD

Background and Purpose—Disturbance of endoplasmic reticulum (ER) Ca^{2+} homeostasis causes neuronal cell injury in stroke. By contrast, ischemic preconditioning (IPC)—a brief sublethal ischemic episode affording tolerance to a subsequent ischemic insult—restores ER Ca^{2+} homeostasis. Under physiological conditions, ER calcium content is continuously refilled by the interaction between the ER-located Ca^{2+} sensor STIM (stromal interacting molecule) 1 and the plasma membrane channel ORAI1 (a structural component of the CRAC calcium channel)—2 key mediators of the store-operated calcium entry (SOCE) mechanism. However, the role played by ORAI1 and STIM1 in stroke and in IPC-induced neuroprotection during stroke remains unknown. Therefore, we explored whether ORAI1 and STIM1 might be involved in stroke pathogenesis and in IPC-induced neuroprotection.

Methods—Primary cortical neurons were subjected to oxygen and glucose deprivation+reoxygenation to reproduce in vitro brain ischemia. Focal brain ischemia and IPC were induced in rats by transient middle cerebral artery occlusion. Expression of ORAI1 and STIM1 transcripts and proteins and their immunosignals were detected by qRT-PCR, Western blot, and immunocytochemistry, respectively. SOCE and Ca^{2+} release–activated Ca^{2+} currents (I_{CRAC}) were measured by Fura-2 AM video imaging and patch-clamp electrophysiology in whole-cell configuration, respectively.

Results—STIM1 and ORAI1 protein expression and immunosignals decreased in the ipsilesional temporoparietal cortex of rats subjected to transient middle cerebral artery occlusion followed by reperfusion. Analogously, in primary hypoxic cortical neurons, STIM1 and ORAI1 transcript and protein levels decreased concurrently with SOCE and Ca^{2+} release–activated Ca^{2+} currents. By contrast, IPC induced SOCE and Ca^{2+} release–activated Ca^{2+} current upregulation, thereby preventing STIM1 and ORAI1 downregulation induced by oxygen and glucose deprivation+reoxygenation. Silencing of STIM1 or ORAI1 prevented IPC-induced tolerance and caused ER stress, as measured by GRP78 (78-kDa glucose regulated protein) and caspase-3 upregulation.

Conclusions—ORAI1 and STIM1, which participate in SOCE, take part in stroke pathophysiology and play an important role in IPC-induced neuroprotection.

Visual Overview—An online visual overview is available for this article. **(*Stroke*. 2019;50:00-00. DOI: 10.1161/STROKEAHA.118.024115.)**

Key Words: calcium homeostasis ■ ischemic preconditioning ■ primary cortical neurons ■ rats ■ stroke

A growing interest has been recently devoted to the role of intracellular Ca^{2+} storage organelles in neuroprotection induced by ischemic preconditioning (IPC)[1,2]—a brief nonlethal ischemic episode that affords tolerance to a subsequent ischemic insult.[3–5] Disturbance of Ca^{2+} homeostasis in the endoplasmic reticulum (ER) represents a common denominator of neuronal cell injury in many neurological disorders, including stroke.[6] Restoring ER Ca^{2+} homeostasis could be a new potential strategy in neurodegeneration. Accordingly, the NCX (Na^+/Ca^{2+} exchanger),[7,8] the Na^+/H^+ exchanger 1,[9] the calcium-binding protein calretinin,[10,11] and the ER chaperone protein GRP78[12] have all been recently identified as molecular mediators of ischemic tolerance in neurons. For instance, severe deficiencies in ER Ca^{2+} refilling and NCX activity under hypoxic conditions in neurons[13,14] can lead to neuronal death. By contrast, increases in NCX reverse mode of operation contribute to IPC-induced Ca^{2+} refilling into the ER, thereby promoting neuroprotection.[8]

Besides Ca^{2+} storage, the ER has an essential function in the Ca^{2+}-dependent folding of newly synthesized proteins.[15,16]

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From the Division of Pharmacology, Department of Neuroscience, Reproductive and Odontostomatological Sciences, School of Medicine, Federico II University of Naples, Italy (A.S., T.P., V.T., F.B., A.V., R.C., A.P., P.M., G.P.); and IRCCS SDN, Naples, Italy (L.A.).

*Drs Secondo and Petrozziello contributed equally.

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Correspondence to Agnese Secondo, PhD, Federico II University of Naples, Via Sergio Pansini 5, Naples 80131, Italy, Email secondo@unina.it or Lucio Annunziato, MD, IRCCS SDN, Via Gianturco, Naples, Italy, Email lannunzi@unina.it

When this function is impaired, as it occurs under hypoxic conditions, unfolded proteins accumulate in the ER lumen, thus determining the activation of specific intracellular pathways. For instance, prolonged Ca^{2+} leak from the ER leads to the activation of GRP78[17] and some transcription factors[18,19] that, in turn, activate the defensive mechanism called unfolded protein response.[6] Prolonged activation of unfolded protein response results in cell death via ER stress.[6] Therefore, severe Ca^{2+} depletion from the ER may account for cell death.[14]

Under physiological conditions, ER Ca^{2+} content is continuously refilled by the interaction between the ER-located Ca^{2+} sensor STIM (stromal interacting molecule) 1 and the plasma membrane channel ORAI1.[20] Cooperating with other partners,[21] these 2 proteins mediate the mechanism called store-operated calcium entry (SOCE), which controls different cell functions.[22–29] On ER Ca^{2+} depletion, STIM1 translocates to the plasma membrane and forms highly Ca^{2+} selective pores with the plasma membrane ORAI1.[30,31] This leads to the Ca^{2+} influx from the extracellular space directly into ER[32–34] through the Ca^{2+} release–activated Ca^{2+} current (I_{CRAC}).

Although well characterized under physiological conditions, the role played by ORAI1 and STIM1 in stroke and in IPC-induced neuroprotection remains completely unknown. In this study, we tested (1) whether hypoxic conditions in primary cortical neurons or transient middle cerebral artery occlusion (tMCAO) might modulate protein expression, interaction, and activity between ORAI1 and STIM1 and (2) whether ORAI1 and STIM1 might be involved in IPC-induced neuroprotection.

Methods

Data, Materials, and Code Disclosure Statement

The data that support the findings of this study are available from the corresponding authors on reasonable request.

Primary Cortical Neurons and siRNA Treatments

Cortical neurons were obtained from brains of 14/16-day-old Wistar rat embryos and dissected as reported previously.[8] After 5/6 days in vitro cortical neurons were transfected with specific siRNAs (#1 or #2) against ORAI1 or STIM1 (Qiagen, Mi, Italy) in order to knock down these proteins. Only siRNAs #2 efficiently reduced ORAI1 or STIM1 protein expression. All animal procedures were performed in accordance with the Animal Care Committee of Federico II University of Naples by Italian Ministerial Authorization (DL 116/92 art.7).

Combined Oxygen and Glucose Deprivation

Preconditioning insult and hypoxia were reproduced in vitro as reported previously.[8]

$[Ca^{2+}]_i$ Measurements

$[Ca^{2+}]_i$ was measured by single-cell computer-assisted video imaging as reported previously.[13,14] For SOCE analysis, neurons were exposed to thapsigargin in the absence of extracellular calcium and, then, re-exposed to 2 mmol/L Ca^{2+}. Nimodipine and ω-conotoxin were added during the recording.[23,35,36]

Electrophysiology

I_{CRAC} (CRAC currents) were recorded in single neurons by patch-clamp in whole-cell configuration at room temperature with continuous perfusion of bath solution. The external solutions contained in mM 145 NaCl, 5 KCl, 1 CaCl2, 1 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4), whereas the pipette solution contained in mM 145 CsCl, 8 NaCl, 10 $MgCl_2$, 10 HEPES, 10 EGTA, and 2 Mg-ATP (pH 7.2). Mg^{2+} and ATP were added to inhibit TRPM7.[37] Nimodipine and spermine chloride were added to block L-type Ca^{2+} channels and Mg^{2+}-inhibitable currents, respectively. Tetraethylammonium and tetrodotoxin were added to the external solution to block tetraethylammonium-sensitive K^+ and tetrodotoxin-sensitive Na^+ currents, respectively. I_{CRAC} and store operated calcium current were recorded with a 100-ms voltage ramp protocol (from +90 to −120 mV) from −15 mV holding potential every 10 s. The magnitudes of I_{CRAC} were measured at the end of −120 mV. Then, 2-APB (30 µmol/L) was added to measure the Ca^{2+} release–activated Ca^{2+}–independent currents. The 2-APB–insensitive components were subtracted from total currents to isolate I_{CRAC}. Capacitive currents were estimated from the decay of capacitive transients induced by 5 mV depolarizing pulses from a holding potential of −80 mV and acquired at a sampling rate of 50 kHz. Furthermore, I_{CRAC} was normalized to membrane capacitance to exclude possible changes in cell size caused by specific treatments (measured as pA/pF).

Determination of Cell Death

Cell death was measured as reported previously.[13]

Transient Focal Ischemia and IPC

Fifty-seven Sprague Dawley male rats (200–225 g; Charles River, Italy), housed under diurnal lighting conditions (12-hour darkness/light), were used. Experiments were performed in a blinded manner. Animal handling was done in accordance with the international guidelines for animal research (ARRIVE). Transient focal ischemia was induced by suture occlusion of middle cerebral artery in male rats anesthetized with a mixture of 2% sevoflurane and 98% O_2 (Oxygen Concentrator Mod. LFY-I-5). Achievement of ischemia was confirmed by monitoring regional cerebral blood flow. Animals not showing cerebral blood flow reduction of at least 70% (n=9), as well as those dying after ischemia induction (n=6), were excluded from the study. For the experiments, rats were divided into 6 experimental groups: (1) sham-operated rats, (2) ischemic rats (tMCAO+vehicle), (3) preconditioned rats (IPC+vehicle), (4) preconditioned rats subjected to tMCAO (IPC+tMCAO+siCTL), (5) preconditioned rats treated with siRNA against ORAI1 and subjected to tMCAO (IPC+tMCAO+siORAI1), and (6) preconditioned rats treated with siRNA against STIM1 and subjected to tMCAO (IPC+tMCAO+siSTIM1). In the tMCAO group the middle cerebral artery was occluded for 100 minutes, whereas in the ischemic preconditioned groups (IPC), middle cerebral artery was occluded for 30 minutes of tMCAO. When IPC precedeed the tMCAO, 72 hours of reperfusion were allowed between the 2 procedures. For siRNA delivery to the rat brain, animals were placed in a stereotaxic frame (David Kopf Instruments). After a hole was made with a stereotaxic-attached drill, a guide cannula (PlasticOne) was fixed to rat skull with dental cement into left lateral ventricle (0.6 mm posterior to bregma, 1.5 mm lateral to the midline, and 3.2 mm beneath the dura). Each siRNA was injected for 5 minutes at the concentration of 5 µmol/L in a volume of 5 µL, 3 hours before preconditioning, and 3 and 18 hours after preconditioning through an infusion cannula connected to a Hamilton syringe. Vehicle group was injected with an untargeted siRNA. Rectal temperature was maintained at 37±5°C with a thermostatically controlled heating pad; arterial blood gases before and after ischemia were measured by a catheter inserted into the femoral artery (Rapid Laboratory 860, Chiron Diagnostic).

Evaluation of the Infarct Volume

The ischemic volume was evaluated with 2,3,5-triphenyl tetrazolium chloride staining, 24 hours after ischemia.[38,39]

Immunocytochemistry

Rats were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally) and perfused transcardially with 4% (w/v) paraformaldehyde.[40] The brains were sectioned coronally at 60 µm. After blocking, sections were incubated with the following primary antibodies: mouse monoclonal anti-NeuN (1:2000; Merk-Millipore, Milan, Italy), polyclonal

anti-ORAI1 (No. ACC-062 1:1000; Alomone Lab, Israel), and polyclonal anti-STIM1 (No. ACC-063 1:1000; Alomone Lab). Sections processed for single-labeling light microscopy were incubated with the corresponding biotinylated secondary antibody, and the peroxidase reaction was developed using 3,3-diaminobenzidine/4-HCl. Sections processed for double immunofluorescence analysis were incubated with the corresponding fluorescent-labeled secondary antibodies (Alexa 488/Alexa 594-conjugated anti-mouse/anti-rabbit IgGs). Controls of the methods in the double immunofluorescence experiments included replacement of the primary antisera with normal serum (1:200). To control for a possible cross-reactivity between IgGs in double immunolabeling experiments, some sections were processed through the same immunocytochemical sequence except that primary antisera were replaced with normal serum or only 1 primary antibody was applied. In addition, all the secondary antibodies were highly preadsorbed to the IgGs of numerous species. Single-labeling light microscopy studies showed ORAI1 and STIM1 distribution on consecutive tissue sections obtained from the same animal groups used in confocal studies. Images were observed using a Zeiss inverted 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) and a 63× oil immersion objective.

Evaluation of mRNA and Protein Levels

The following sequences of primers were used for real-time polymerase chain reaction (7500fast; Applied Biosystems)[41]:

Orai1 5′-TGGCCGTGCACCTGTTC-3′ and 5′-TTGCTCACAG CCTCGATGTT-3′; *Stim1* 5′-TCAGCGTGGAGGACCTATGG-3′ and 5′-CACATACGTAATCAGCCACTGTATCA-3′; *HPRT* 5′-TCCATTC CTATGACTGTAGATTTTATCAG-3′ and 5′-AACTTTTATGTCCC CCGTTGACT-3′.

For protein expression analysis, ice-cold lysis buffer contained 20 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 1% NONIDET P-40, 1 mmol/L Na_3VO_4, 0.1% aprotinin, 0.7 mg/mL pepstatin, and 1 µg/mL leupeptin. Protein concentration was determined by the Bradford method[42]; samples were separated on 10% SDS-polyacrylamide gels and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Milan, Italy). Membranes were blocked and incubated overnight at 4°C with rabbit polyclonal anti-GRP78 (No. 3183, 1:1000; Cell Signaling Technology, Inc, Danvers, MA) or rabbit polyclonal anti–caspase-3 (No. 9661, 1:1000; Cell Signaling Technology, Inc) or polyclonal anti-ORAI1 (No. ACC-062 1:1000; Alomone Lab) or polyclonal anti-STIM1 (No. ACC-063 1:1000; Alomone Lab) or TRPC1 (transient receptor potential channel type 1; No. ACC-118 1:1000; Alomone Lab).

Proximity Ligation Assay

Cortical neurons plated on glass coverslips were fixed at room temperature in 4% (w/v) paraformaldehyde. Afterward, cells were blocked and incubated overnight at 4°C with monoclonal anti-ORAI1 (No. SAB3500126 1:1000; Sigma-Aldrich, Milan, Italy) and polyclonal anti-STIM1 (No. ACC-063 1:1000; Alomone Lab). Next, cells were visualized with the Red Duolink kit (Sigma-Aldrich).

Statistical Analysis

All data sets are presented as mean±SE. One-way ANOVA and post hoc test with Bonferroni correction or Newman-Keuls correction were performed to compare >2 groups. To compare data sets from 2 groups, parametric unpaired Student *t* test was performed. Differences were considered statistically significant when the P was <0.05.

Results

STIM1 and ORAI1 Levels in the Ipsilesional Temporoparietal Cortex of Rats Subjected to tMCAO and in Hypoxic Cortical Neurons

The role of ORAI1 and STIM1 in focal ischemia was determined by subjecting adult rats to tMCAO followed by 24

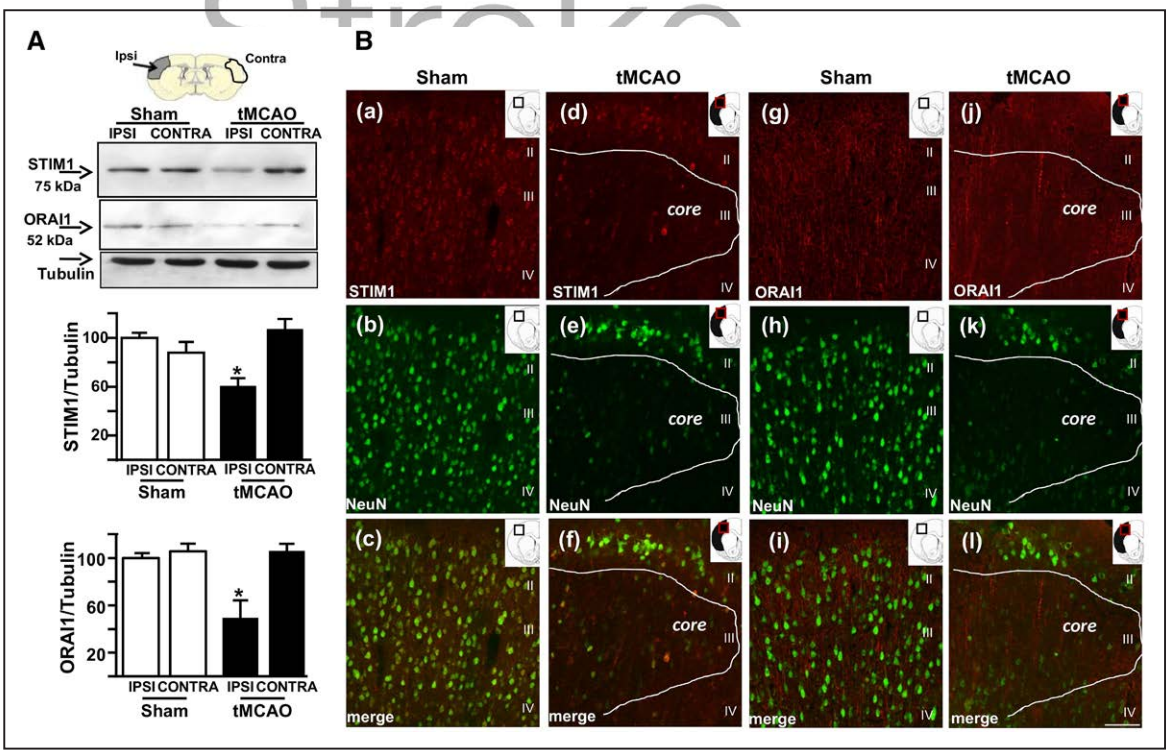

Figure 1. STIM1 (stromal interacting molecule 1) and ORAI1 (a structural component of the CRAC calcium channel) expression and immunoreactivity in the temporoparietal cortex of rats subjected to transient middle cerebral artery occlusion (tMCAO). **A**, STIM1 and ORAI1 proteins in ipsilateral (IPSI) and contralateral (CONTRA) cortices of rats subjected to tMCAO and of sham-operated animals. Data were normalized on the tubulin levels and expressed as percentage of the relative protein expression in IPSI sham-operated rats. n=8 animals for each column. Values are mean±SE. **P*<0.05 vs all. **B**, STIM1 (**Ba**–**Bf**) and ORAI1 (**Bg**–**Bl**) immunoreactivity in temporoparietal cortex in sham-operated animals (**Ba**–**Bc** and **Bg**–**Bi**) and in tMCAO rats (**Bd**–**Bf** and **Bj**–**Bl**). STIM1/NeuN (neuronal nuclei) (**Bc** and **Bf**) and ORAI1/NeuN (**Bi** and **Bl**) colocalization in IPSI cortex of ischemic rats. Scale bars=50 µm.

hours of reperfusion. STIM1 and ORAI1 protein expression was significantly reduced in the ipsilesional temporoparietal cortex compared with the corresponding region of sham-operated animals (Figure 1A). Furthermore, confocal double labeling immunofluorescence experiments showed that both STIM1 and ORAI1 were abundantly coexpressed in the NeuN-positive cells of the temporoparietal cortex of sham-operated animals (Figure 1B). Whereas STIM1 immunosignal was mainly distributed within the soma (Figure 1Ba through 1Bc), ORAI1 immunoreactivity was also detected along neuronal processes (Figure 1Bg through IBi). By contrast, STIM1 and ORAI1 immunosignals were concomitantly reduced in the ipsilesional temporoparietal cortex of ischemic animals (Figure 1Bd through 1Bf and 1Bj–1Bl, respectively). Furthermore, in the perilesional cortical region of ischemic rats, where ongoing neuronal degeneration can be observed (Figure I in the online-only Data Supplement), STIM1 immunoreactivity appeared globally reduced, although some scattered cells were intensely labeled (Figure ID–IF in the online-only Data Supplement). Similarly, anti-ORAI1 antibody stained neuronal soma less intensely than in control slices (Figure IG and IJ in the online-only Data Supplement), while occasional neurites showed pronounced labeling (Figure IJ through IL in the online-only Data Supplement).

Exposure of cortical neurons to oxygen and glucose deprivation (OGD) for 3 hours dramatically reduced STIM1 and ORAI1 transcripts and proteins (Figure 2Aa and 2Ab and 2Ba through 2Bc). Twenty-one hours after reoxygenation, the transcript levels of STIM1 and ORAI1 but not the proteins returned to pretreatment levels (Figure 2A and 2B). By contrast, the expression of TRPC1—the main SOCE partner of ORAI1 and STIM1[43]—did not change (Figure IIA in the online-only Data Supplement). Accordingly, TRPC1-mediated store operated calcium current[43] did not change in hypoxic neurons (Figure IIB in the online-only Data Supplement). In the presence of siRNA against ORAI1 or STIM1 (Figure IIC in the online-only Data Supplement, top), SOCE was significantly reduced (Figure IIC in the online-only Data Supplement, bottom). Similarly, the voltage step protocol (hyperpolarized to −120 from −15 mV) revealed that I_{CRAC} was also reduced (Figure IID in the online-only Data Supplement). In hypoxic neurons, SOCE-mediated Ca^{2+} entry and I_{CRAC} activation were significantly reduced (Figure 2C and 2D).

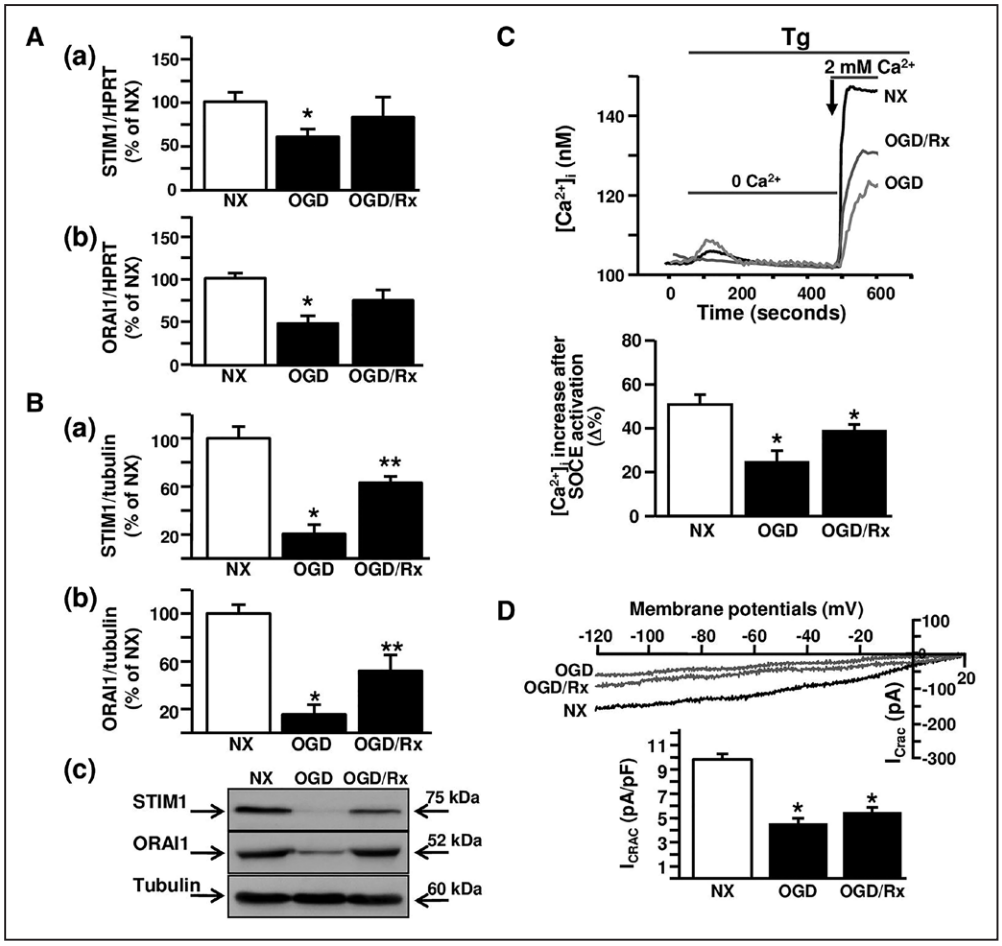

Figure 2. Effect of oxygen and glucose deprivation (OGD) on ORAI1 (a structural component of the CRAC calcium channel) and STIM1 (stromal interacting molecule 1) transcripts and proteins, on store-operated calcium entry (SOCE), and Ca^{2+} release–activated Ca^{2+} current (I_{CRAC}) in primary cortical neurons. **A**, STIM1 and ORAI1 transcripts (**Aa** and **Ab**) in cortical neurons exposed to 3 h of OGD and OGD/reoxygenation (Rx). Data are means±SE of 4 separate experiments. **P*<0.05 vs normoxic (NX) cells. **B**, Western blot of STIM1 and ORAI1 expression (**Ba**–**Bc**) in cortical neurons exposed to OGD and OGD+Rx. Data are means±SE of 4 separate experiments. **P*<0.05 vs NX. ***P*<0.05 vs all. **C**, Traces (**top**) and quantification (**bottom**) of the effect of OGD or OGD+Rx on SOCE. Data are means±SE of 3 separate experiments (30–50 cells for each experiment). **P*<0.05 vs NX. **D**, Whole-cell recordings and quantification of I_{CRAC} in cortical neurons. Data are means±SE of 3 separate experiments (5–8 cells for each group). **P*<0.05 vs NX.

STIM1 and ORAI1 Expression in Temporoparietal Cortex of Preconditioned Adult Rats and Preconditioned Cortical Neurons

Seventy-two hours after tolerance induction, single-labeling light microscopy revealed that STIM1 and ORAI1 were upregulated in the ipsilateral temporoparietal cortex of preconditioned rats (Figure III in the online-only Data Supplement). Furthermore, colocalization experiments revealed that both STIM1 and ORAI1 were intensely upregulated within NeuN-positive neurons (Figure 3A and 3B). STIM1 immunosignal was intensely detected within the neuronal soma (Figure 3Ad through 3Af), whereas upregulation of ORAI1 was observed not only in the soma but also within a large number of processes (Figure 3Bd through 3Bf). Interestingly, 72 hours after tolerance induction, STIM1 immunoreactivity also became clearly evident in scattered perineuronal cells resembling glial morphology (see arrows in Figure 3Af). Moreover, STIM1 and ORAI1 expression peaked in primary cortical neurons exposed to IPC followed by 1 hour of normoxia but returned to the control level at later time points (Figure 3C and 3D). The in situ proximity ligation assay showed that there was an increase of contiguity between STIM1 and ORAI1 in preconditioned cortical neurons (Figure 4A). More interestingly, this interaction was higher during IPC than in the presence of the sarco/endoplasmic reticulum Ca(2+) ATPase inhibitor thapsigargin (Figure 4A). In accordance with these results, IPC enhanced SOCE, whereas siSTIM1 or siORAI1 prevented it (Figure 4Ba and 4Bb). Moreover, siSTIM1 or siORAI1 prevented ER Ca^{2+} accumulation induced by IPC (Figure 4Bc). Accordingly, I_{CRAC} was significantly increased in neurons

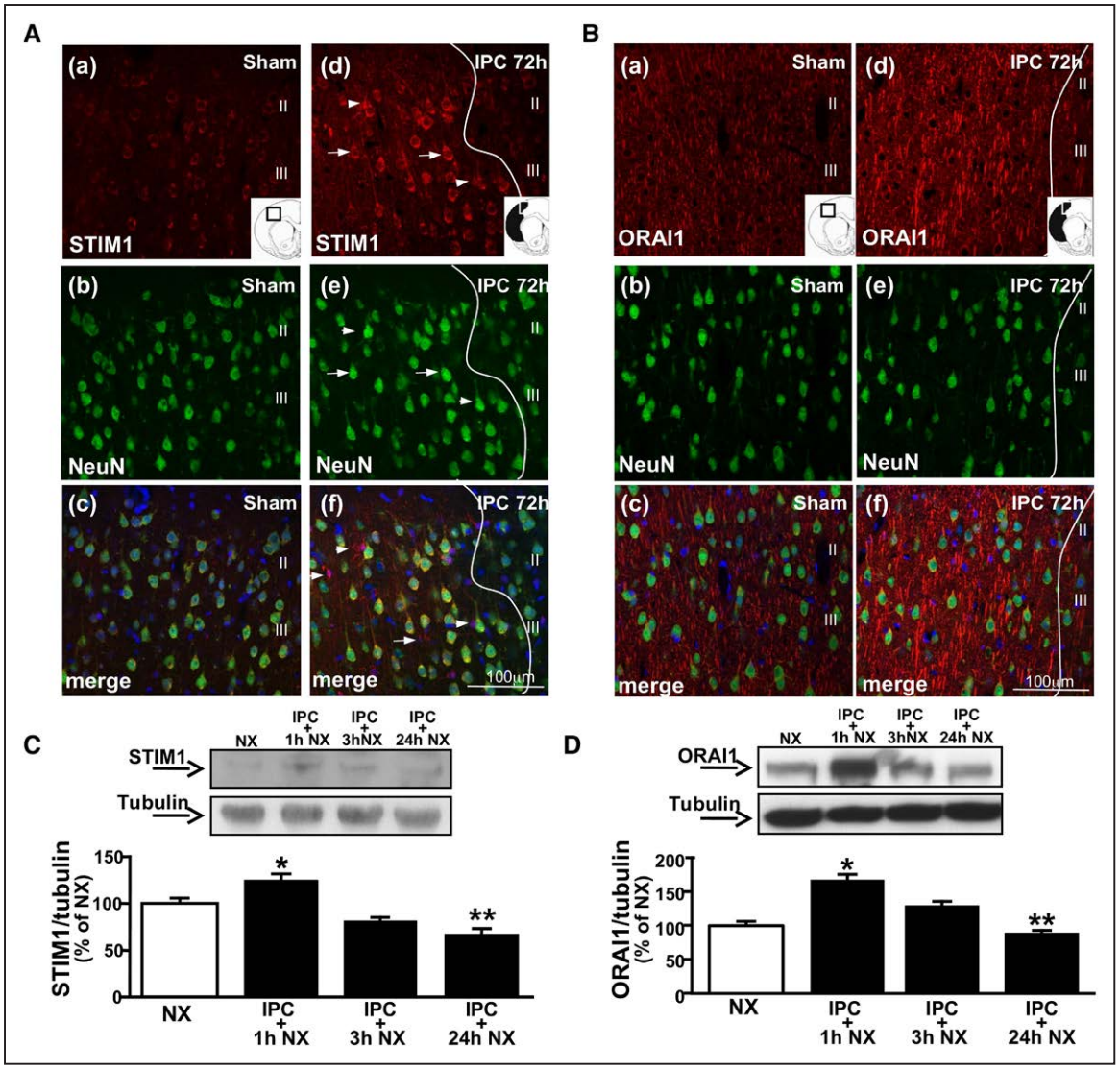

Figure 3. STIM1 (stromal interacting molecule 1) and ORAI1 (a structural component of the CRAC calcium channel) immunoreactivity in the ipsilateral temporoparietal cortex of preconditioned rats, and STIM1 and ORAI1 interaction in preconditioned primary cortical neurons. **A**, STIM1 immunoreactivity in the rat cerebral cortex in sham-operated animals (**Aa**–**Ac**) and in preconditioned rats (**Ad**–**Af**). STIM1/NeuN (neuronal nuclei) colocalization in ipsilateral cortex of sham-operated (**Ac**) and preconditioned rats (**Af**). Arrows in **Af** indicate scattered perineuronal cells. **B**, ORAI1 immunoreactivity in the rat cerebral cortex in sham-operated animals (**Ba**–**Bc**) and in preconditioned rats (**Bd**–**Bf**). ORAI1/NeuN colocalization in ipsilateral cortex of sham-operated (**Bc**) and preconditioned rats (**Bf**). Dashed lines delimit preconditioned from normal tissue. **C** and **D**, Western blots of STIM1 (**C**) and ORAI1 (**D**) expression in cortical neurons under normoxic conditions or after exposure to ischemic preconditioning (IPC). Data are means±SE of 3 separate experiments. **P*<0.05 vs normoxia (NX), IPC +3 h and IPC +24 h. ***P*<0.05 vs NX and IPC +1 h.

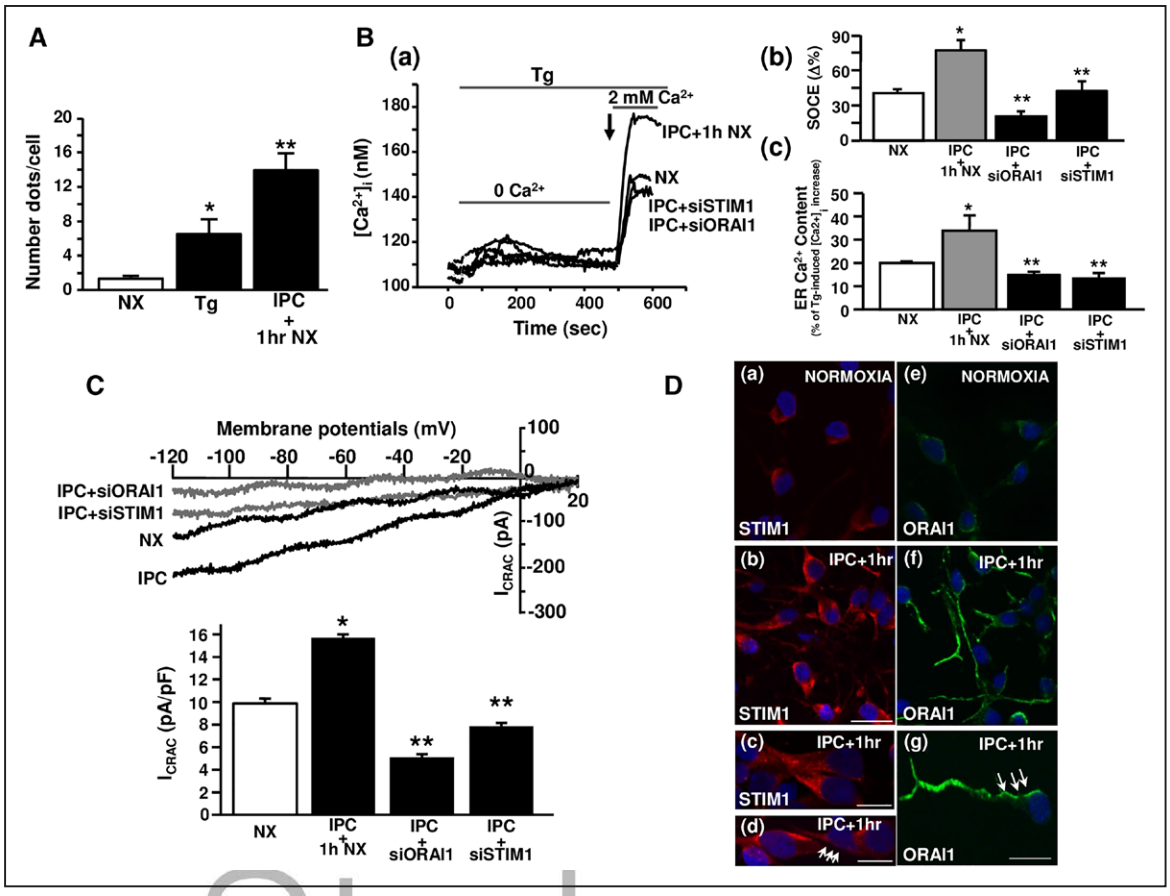

Figure 4. Effect of ischemic preconditioning (IPC) on store-operated calcium entry (SOCE) and Ca^{2+} release–activated Ca^{2+} current (I_{CRAC}) in primary cortical neurons. **A**, STIM1 (stromal interacting molecule 1) and ORAI1 (a structural component of the CRAC calcium channel) interaction in cortical neurons under normoxic conditions, after thapsigargin (Tg, 30 min) and after IPC +1 h normoxia (NX) measured as number of total red dotted puncta. **P*<0.05 vs NX; ***P*<0.05 vs all. **B**, Traces (**Ba**) and quantification (**Bb**) of the effect of IPC +1 h NX on SOCE-mediated $[Ca^{2+}]_i$ increase in the absence or presence of siORAI1 or siSTIM1 (10 nmol/L for 48 h); (**Bc**) Quantification of the effect of IPC +1 h NX on ER Ca^{2+} content measured as $[Ca^{2+}]_i$ increase after thapsigargin addition. Data are means±SE of 3 separate experiments (40 cells for each experiment). **P*<0.05 vs normoxic cells. ***P*<0.05 vs IPC. **C**, Whole-cell recordings (**top**) and quantification (**bottom**) of I_{CRAC} in cortical neurons under NX and after IPC +1 h NX in the absence or presence of siORAI1 or siSTIM1 (10 nmol/L for 48 h). Data are means±SE of 3 separate experiments (5–8 cells for each group). **P*<0.05 vs respective normoxic cells. ***P*<0.05 vs IPC +1 h. **D**, STIM1 (**Da–Dd**) and ORAI1 (**De–Dg**) immunoreactivity in cortical neurons under normoxic conditions (**Da** and **De**) or after IPC +1 h NX (**Db–Dd**, **Df**, and **Dg**).

exposed to IPC compared with normoxic neurons, whereas siSTIM1 or siORAI1 prevented IPC-induced increase of I_{CRAC} (Figure 4C). Finally, in preconditioned neurons, STIM1 antibody clearly depicted a more pronounced immunoreactivity within the neuronal soma (Figure 4Da through 4Dd) and displayed a punctuate staining, which was also associated with a plasma membrane distribution (Figure 4Db through 4Dd). Moreover, the upregulation of ORAI1 immunosignal was detected along the plasma membrane of both soma and processes of IPC neurons (Figure 4Df and 4Dg).

Knocking Down STIM1 or ORAI1 Expression on IPC-Induced Neuroprotection

The reduction in STIM1 and ORAI1 protein expression in the ipsilesional cortex of rats subjected to tMCAO was prevented by IPC (Figure 5A and 5B). Interestingly, IPC determined per se a significant upregulation of both proteins in the same brain region (Figure 5A and 5B). Therefore, rats subjected to IPC plus harmful ischemia were intracerebroventricularly (icv) injected with selective siRNAs against both proteins. Each icv siRNA treatment significantly reverted IPC-induced neuroprotection and IPC-induced improvement of behavioral deficits (Figure 5C and 5D).

In vitro, OGD+RX induced a depletion of ER Ca^{2+} stores while IPC restored ER Ca^{2+} content (Figure 6A). Interestingly, siSTIM1 or siORAI1 prevented IPC-dependent restoration of ER Ca^{2+} content (Figure 6A). Furthermore, oxygen and glucose deprivation+reoxygenation increased GPR78 (BiP) and active caspase-3 protein expression (Figure 6Ba and 6Bb). However, such effect was prevented by IPC (Figure 6B). In contrast, siORAI1 or siSTIM1 significantly counteracted the reduction in GPR78 (Figure 6Ba) and active caspase-3 induced by IPC (Figure 6Bb). Moreover, cell death induced by oxygen and glucose deprivation+reoxygenation was prevented by IPC (Figure 6C and 6D), whereas IPC-induced neuroprotection was reverted by siORAI1 or siSTIM1 (Figure 6C and 6D).

Discussion

The present study has demonstrated for the first time that ORAI1 and STIM1 are involved in the pathophysiology of stroke and in the mechanisms of ischemic tolerance. Indeed ORAI1 and STIM1 level was intensely downregulated in the

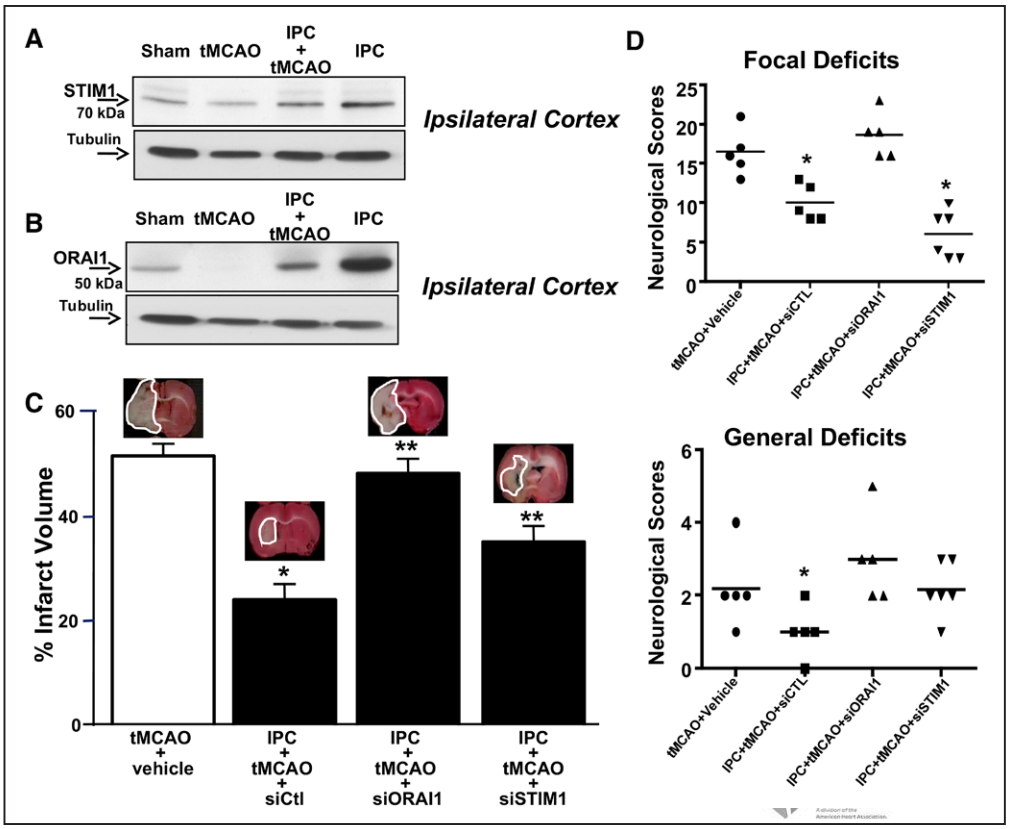

Figure 5. Effect of siSTIM1 and siORAI1 on ischemic preconditioning (IPC)–induced tolerance in rats. **A** and **B**, Western blots of STIM1 (stromal interacting molecule 1; **A**) and ORAI1 (a structural component of the CRAC calcium channel) (**B**) in the ipsilateral cortex of sham-operated, ischemic, and preconditioned rats (±transient middle cerebral artery occlusion [tMCAO]). Values are mean±SE. **C**, Infarct volume in rats subjected to tMCAO+vehicle, IPC+tMCAO+siCTL (untargeted siRNA used as control), IPC+tMCAO+siORAI1 (siRNA against ORAI1), and IPC+tMCAO+siSTIM1 (siRNA against STIM1). Each column represents the percent ratio between the volumes of the hemispheres ipsilateral and contralateral to tMCAO in each group.*P<0.05 vs tMCAO; **P<0.05 vs IPC+tMCAO. **D**, Neurological deficits in rats subjected to the same treatments of **C**. *P<0.05 vs tMCAO+vehicle (**top**); *P<0.05 vs all (**bottom**).

NeuN-positive cells of the ischemic core of rats subjected to tMCAO. Similarly, their expression and activity were significantly reduced in hypoxic cortical neurons.

Another interesting finding of our study is that IPC per se not only triggered the upregulation of ORAI1 and STIM1 expression, activity, and physical interaction but also prevented their downregulation in primary hypoxic cortical neurons and in the ipsilesional cortex of rats subjected to tMCAO. Furthermore, the silencing of ORAI1 or STIM1 significantly counteracted the neuroprotection mediated by IPC in primary hypoxic neurons and in rats subjected to tMCAO.

Furthermore, under low oxygen conditions, IPC (1) increased ORAI1 and STIM1-mediated Ca^{2+} refilling into ER, (2) restored ER Ca^{2+} homeostasis, and (3) suppressed the upregulation of ER stress markers, that is, GRP78 and caspase-3.

That ORAI1 and STIM1 upregulation occurred after preconditioning in those brain regions that were protected from subsequent ischemic injury further supports the hypothesis that ORAI1 and STIM1 upregulation constitutes a newly identified cell-protective response against stroke. We speculate that neuroprotection mediated by STIM1 and ORAI1 overexpression rely to the restoration of Ca^{2+} homeostasis via ER Ca^{2+} refilling with subsequent ER stress prevention and reduced cell death. These data are in accordance with our and other previous studies demonstrating (1) the detrimental role played by dysfunctional ER Ca^{2+} homeostasis in hypoxic cortical neurons[14] and in the ischemic core[44] and (2) the protective role of Ca^{2+} refilling in the ER as an early event occurring in IPC.[8] Several ER proteins are functionally coupled to STIM1/ORAI1 complex. In fact, the sarco/endoplasmic reticulum Ca^{2+} ATPase pump colocalizes with STIM1 at the subplasmalemmal region favoring rapid Ca^{2+} extrusion after SOCE activation.[45] Furthermore, translocation of IP3Rs to ER–plasma-membrane junctions facilitates STIM/ORAI coupling leading to SOCE activation.[46] Although many ligands of RyRs are able to modulate SOCE through a direct interaction with ORAI, the knowledge on a direct involvement of RyR in STIM1/ORAI1 function still lack. On the contrary, the functional association between RyR1 and TRPC1 channel has been demonstrated.[47] However, how such ER proteins intervening in this complex can take part in SOCE during stroke or IPC remains unknown.

Interestingly, the increase in ORAI1 and STIM1 expression and activity occurring in preconditioned primary cortical neurons was transient and temporally short. On the contrary, the long-lasting neuroprotection observed after induction of IPC suggests that other players may well intervene in the later phases of stroke to maintain ER Ca^{2+} refilling, thereby facilitating neuroprotection during subsequent lethal insults. This

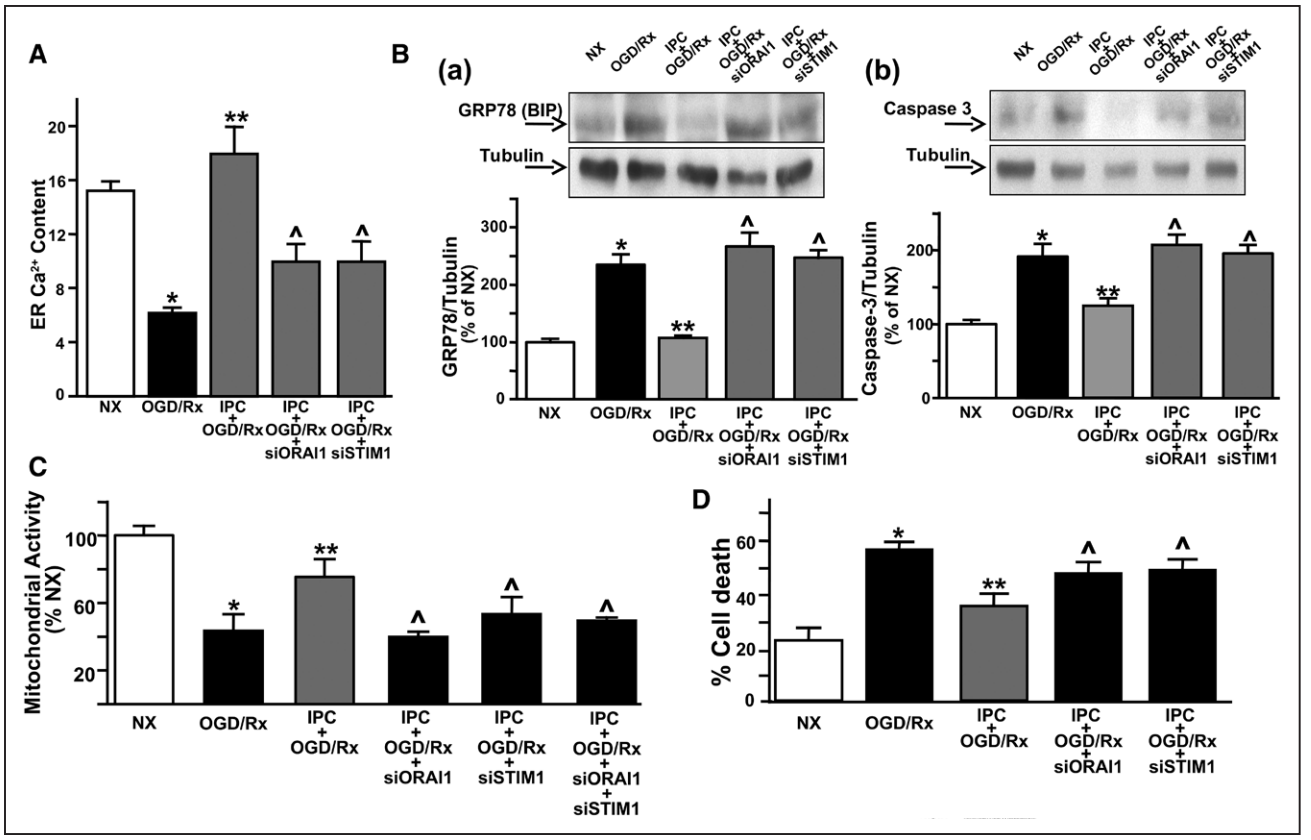

Figure 6. Effect of siSTIM1 or siORAI1 on ischemic preconditioning (IPC)–induced neuroprotection and ER stress in cortical neurons. **A**, Effect of oxygen and glucose deprivation (OGD)/reoxygenation (Rx) and IPC followed by OGD/Rx on ER Ca^{2+} content in the absence or presence of siORAI1 or siSTIM1 (10 nmol/L per 48 h). Data are means±SE of 3 independent experiments. *P<0.05 vs normoxia (NX); **P<0.05 vs OGD/Rx; ^P<0.05 vs IPC+OGD/Rx. **Ba** and **Bb**, Western blot of GRP78 (78-kDa glucose regulated protein) (**Ba**) and active caspase-3 (**Bb**) in cortical neurons under the conditions mentioned in **A**. Data are means±SE of 5 separate experiments. *P<0.05 vs NX; **P<0.05 vs OGD/Rx; ^P<0.05 vs IPC+OGD/Rx. **C** and **D**, Mitochondrial activity and cell death measured in cortical neurons under the conditions mentioned in **A**. Data are means±SE of 3 separate experiments. *P<0.05 vs NX; **P<0.05 vs OGD/Rx; ^P<0.05 vs IPC+OGD/Rx.

long-lasting effect could also imply a possible amplification of some signaling cascades mediating ischemic tolerance at the neuronal level. Indeed, silencing of STIM1 and ORAI1 prevented the protective effect of preconditioning after 48 hours. Lastly, because ORAI1 promotes the activation of the protective NFAT (nuclear factor–activated T cells),[48,49] it is plausible that this plasma membrane channel also contributes to IPC-induced neuroprotection by promoting the activation of this transcription factor. As concerns other mechanisms regulating ORAI1 and STIM1 levels during the ischemic process, the expression of both proteins is modulated by NF-κB[50]—a transcriptional factor mainly involved in determining neuronal fate during stroke[51] and IPC.[52] Furthermore, also Akt plays a regulatory role in ORAI1 expression and SOCE.[53] Interestingly, IPC promotes phosphorylation of Akt at Ser473 regulating both caspase-dependent and caspase-independent cell death.[54]

Furthermore, the ubiquitin-like protein ubiquilin-1 colocalizes with the plasma membrane channel facilitating its degradation and thus reducing SOCE.[55] This degradation is efficiently inhibited by bafilomycin-A but not by the proteasome inhibitor MG132, suggesting a lysosomal degradation of ORAI1.[55] Interestingly, ubiquilin-1–dependent ubiquitination[56] and lysosomal-dependent degradation[57] are 2 important processes active during stroke.

Finally, this study demonstrates that ORAI1 and STIM1 are 2 important mediators of preconditioning-mediated tolerance and should, therefore, be considered as new relevant targets in stroke intervention.

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Drs Secondo and Annunziato contributed to the conception and design of the study. Dr Secondo performed the video imaging experiments. Dr Petrozziello performed cell cultures and Western blot. Drs Petrozziello and Tedeschi measured cell death. Dr Boscia performed immunohistochemistry. Dr Molinaro performed qRT-PCR (quantitative real time PCR). Drs Vinciguerra and Pignataro performed transient middle cerebral artery occlusion. Drs Ciccone and Pannaccione contributed to patch clamp. Drs Annunziato and Secondo edited the manuscript.

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Disclosures

None.

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