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Tolerance Induced by (S)-3,5-Dihydroxyphenylglycine Postconditioning is Mediated by the PI3K/Akt/GSK3β Signalling Pathway in an *In Vitro* Model of Cerebral Ischemia

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Abstract—Ischemic postconditioning (PostC) is an endogenous neuroprotective strategy for cerebral ischemia induced by low activation of glutamate receptors. We have previously shown that the application of the mGluR1/5 agonist (S)-3,5-dihydroxyphenylglycine (DHPG) 5 min after 30 min of oxygen and glucose deprivation (OGD) reduces CA1 damage in organotypic hippocampal slices by activating the PI3K–Akt signalling pathway. In order to extend these data, we analysed the production of reactive oxygen species (ROS) and the glycogen synthase kinase 3β (GSK3β) signalling pathway. Our results show that DHPG PostC was associated with a reduction in the formation of ROS that is massively increased 24 h after OGD exposure. This reduction was prevented by the PI3K inhibitor LY294002, indicating that there is a link between the PI3K/Akt pathway and the formation of ROS in the protective mechanisms of PostC. DHPG PostC also induces a transient increased in GSK3β phosphorylation and inactivation that is followed by nuclear accumulation of β-catenin, that probably lead to the up-regulation of neuroprotective genes. Our results propose GSK3β as new target for neuroprotection, therefore, we verified that the two GSK3β inhibitors N-(3-Chloro-4-methylphenyl)-5-(4-nitrophenyl)-1,3,4-oxadiazol-2-amine (TC-G 24) and LiCl are neuroprotective agents in OGD and also can be used as PostC agents. © 2020 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: DHPG postconditioning, oxygen and glucose deprivation, PI3K/Akt/GSK3β signaling pathway, organotypic hippocampal slices, Lithium chloride.

INTRODUCTION

Ischemic postconditioning (PostC) has been established as a novel neuroprotective strategy against ischemic stroke and is defined as a single or a series of brief interruptions in the cerebral blood flow supply performed immediately after a severe ischemic insult (Zhao et al., 2012). However, the molecular mechanisms responsible for neuroprotection induced by PostC remain largely unclear. In a previous paper of our laboratory, we have showed that it is possible to evoke a neuroprotective PostC response in organotypic hippocampal slices exposed to 30 min OGD by applying, 5 min later, a relatively low dose of the mGluR1/5 agonist 3,5-dihydroxyphenylglycine (DHPG) (Scartabelli et al., 2008). This neuroprotection is mediated by the activation of the mGlu1/mGlu5–PI3K–Akt signaling pathway and can be completely abolished by inhibitors of PI3K and Akt activity (Scartabelli et al., 2008). However, our understanding of the mechanistic pathways linking DHPG PostC and neuroprotection remains incomplete.

It has been largely demonstrated that after an ischemic insult, the production of reactive oxygen species (ROS) increases and leads to tissue damage (Liu et al., 2003). Despite this, ROS have been paradoxically implicated as an important signaling component in neuroprotective pathways activated by preconditioning PreC (Nie et al., 2006), PostC (Tsutsumi et al., 2007) and remote ischemic postconditioning (RPostC) (Wang et al., 2011). Oxidative stress that follows reperfusion is related to the enormous generation of ROS and is considered injurious to cell functions and mitochondria (Song et al., 2013). The protective effects of PostC in ischemia/reperfusion injury is not only associated with inhibition of inflammation and apoptosis (Xing et al., 2008a,b), but also with attenuation of oxidative stress (Wang et al., 2008; Ren et al., 2008).

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Abbreviations: CREB, cAMP response element-binding; DHPG, (S)-3,5-dihydroxyphenylglycine; GSK3β, glycogen synthase kinase 3β; OGD, oxygen and glucose deprivation; PostC, postconditioning; PreC, Preconditioning; ROS, reactive oxygen species; TC-G 24, *N*-(3-Chloro-4-methylphenyl)-5-(4-nitrophenyl)-1,3,4-oxadiazol-2-amine.

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The PI3K/Akt pathway has been indicated to play a critical role in survival in PreC (Gerace et al., 2012b; Landucci et al., 2016) and PotsC (Ye et al., 2012). Zhou and colleagues (Zhou et al., 2011) have also shown that phosphorylation (ser 9) and subsequent inactivation of glycogen synthase kinase 3β (GSK3β) via the PI3K/Akt pathway is an important element in the neuroprotective effects of delayed ischemic PostC against global cerebral ischemia. GSK3β is not only a downstream target of Akt, but is also involved in the Wnt pathway (Cook et al., 1996). GSK-3 inactivation has been proposed as a mechanism to promote neuronal survival (Liang and Chuang, 2007). In fact, when GSK3β is inactivated, it leads to β-catenin accumulation and translocation from the cytosol to the nucleus (Ding et al., 2000). β-Catenin mediates anti-apoptotic effects by regulating the expression of several important anti-apoptotic genes such as fibronectin, cyclin DI, c-myc and CaMKIV (Gordon and Nusse, 2006). Increased intracellular calcium levels trigger a series of events that involve not only CaMKIV gene expression, but also cAMP response element-binding (CREB) protein phosphorylation that promote survival through the transcription of CREB-dependent genes, such as c-fos (Gallin and Greenberg, 1995). Lithium as well as other GSK-3β inhibitors has neuroprotective effects against neurological disorders. Delayed lithium treatment provided neuroprotection against focal (Taliyan and Ramagiri, 2016) and global ischemia (Bian et al., 2007).

The aim of the present study was to examine the hypothesis that the protection afforded by DHPG PostC is mediated via the PI3K/Akt/GSK3β signaling pathway in rat organotypic hippocampal slices.

EXPERIMENTAL PROCEDURES

Male and female Wistar rat were obtained from Charles River (MI, Italy). Animals were housed at 23 ± 1 °C under a 12 h light–dark cycle (lights on at 07:00) and were fed a standard laboratory diet with ad libitum access to water. Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

The experimental protocols were approved by the Animal Care Committee of the Department of Health Sciences, University of Florence, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123) and the European Communities Council Directive of 2010/63/EU. The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

Materials

DHPG was purchased from Abcam plc (Cambridge, UK). *N*-(3-Chloro-4-methylphenyl)-5-(4-ni-trophenyl)-1,3,4-oxadiazol-2-amine (TC-G 24) was purchased from Tocris Bioscience (Bristol, United Kingdom, UK). Propidium iodide (PI), lithium chloride and resveratrol were purchased from Sigma (St Louis, MO, USA). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was obtained from Calbiochem (Merck Biosciences Ltd, Nottingham, UK). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma (St Louis, MO, USA).

Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Gerace et al., 2012a). Briefly, transverse hippocampal slices (420 mm) were obtained from 7 to 9 days old Wistar rats and were prepared using a McIlwain tissue chopper. The integral slices were selected and then cultured onto semiporous membrane inserts (Millicell-CM; Millipore, Italy) placed in six well tissue culture plates containing 1.2 ml medium per well (50% Eagle's minimal essential medium, 25% heat-inactivated horse serum, 25% Hanks' balanced salt solution, 5 mg/ml glucose, 2 mM L-glutamine, and 3.75 mg/ml amphotericin B). Slices were kept in culture for 14 days and at 37 °C in an incubator in atmosphere of humidified air and 5% CO_2. Before experiments all slices were screened for viability by phase-contrast microscopy analysis; slices displaying signs of neurodegeneration were discarded from the study.

OGD and postconditioning protocols in rat organotypic hippocampal slices

Cerebral ischemia *in vitro* was mimicked by exposing hippocampal cultures to 30 min of oxygen and glucose deprivation (OGD) as previously reported in detail (Gerace et al., 2015). Following 30 min of incubation at 37 °C in an anaerobic chamber (BioSpherix, New York, USA), the cultures were transferred to oxygenated serum-free medium and returned to the incubator under normoxic conditions. Neuronal injury was evaluated 24 h later. For postconditioning experiments, organotypic hippocampal slices were exposed firstly to 30 min OGD and 5 min later to 10 μM DHPG for 30 min (Scartabelli et al., 2008).

Assessment of CA1 pyramidal cell injury

Cell injury was assessed by using the fluorescent dye propidium iodide (PI, 5 μg/ml). Fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, United Kingdom) equipped with a xenon-arc lamp, a low-power objective (4×) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, United States) controlled by software (InCyt Im1TM; Intracellular Imaging Inc., Cincinnati, OH, United States) and subsequently analysed using a morphometric analysis software Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, United States). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image

software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected.

Reactive oxygen species (ROS) production

Oxidant-sensing fluorescent probe, 2,7-dichlorofluorescein diacetate (DCFH2-DA) was used to detected the formation of intracellular peroxides in organotypic hippocampal slices (Liu et al., 2003). Organotypic hippocampal slices were incubated with 10 μM DCFH2-DA at 37 °C for 30 min. Fluorescence was viewed 30 min later using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp and a rhodamine filter (excitation 450 and 490 nm, emission 520 nm). Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analyzed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify ROS production, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of DCFH2-DA fluorescence was detected (Boscia et al., 2006).

Western blot analysis

Western blotting experiments were performed as previously reported in (Landucci et al., 2016). Briefly,

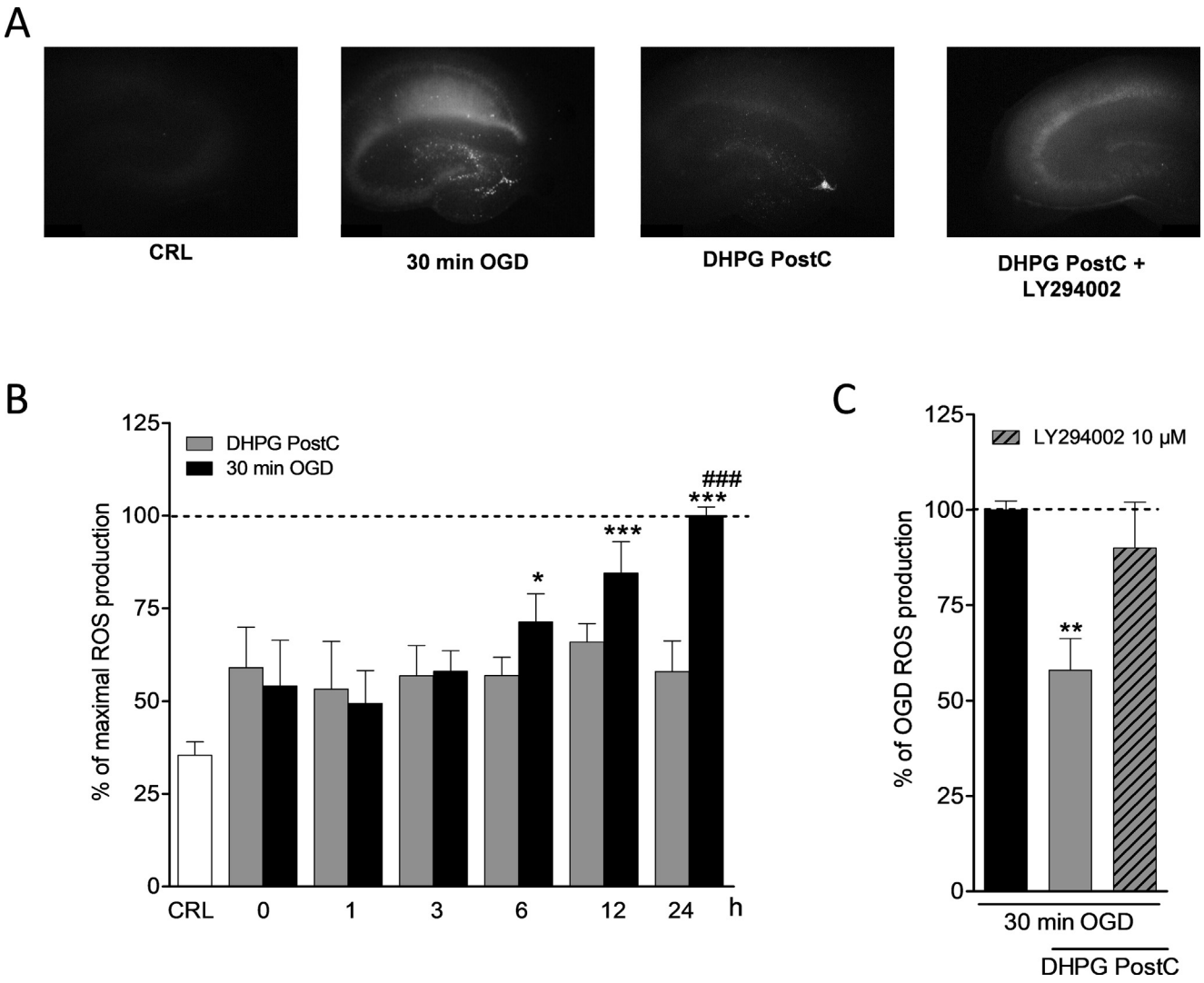

Fig. 1. DHPG PostC induces neuroprotection against OGD injury by reducing the formation of ROS through PI3K pathway. **(A)** Under control conditions, organotypic hippocampal slices display background DCF fluorescence. 24 h after DHPG PostC the slices displayed a marked reduction of neuronal injury in the CA1 subregion induced by 30 min OGD. This reduction is reverted by the incubation with LY294002 during DHPG PostC and during the subsequent 24 h. **(B)** Bars represent the quantitative analysis of CA1 DCF fluorescence and show that OGD induced a time-dependent increase in ROS formation. This increase is significantly reduced by DHPG PostC. **(C)** Quantitative analysis showing that the toxicity produced by OGD was reduced by DHPG PostC and this reduction was prevented by LY294002. Data are expressed as percentage of OGD-induced ROS production in CA1 region. Bars represent the mean ± SEM of at least four experiments run in quadruplicate. **(B)** $^{*}p < 0.05$, $^{***}p < 0.001$ vs. CRL; $^{\#\#\#}p < 0.001$ DHPG PostC vs. OGD 24 h. **(C)** $^{**}p < 0.01$ vs. OGD (ANOVA + Tukey's *w* test).

cultured slices were dissolved in 1% SDS and protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Lysates were resolved by electrophoresis on a 4–20% Tris Glycine Gel (Invitrogen, San Giuliano Milanese, Italy) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, CA, USA). After blocking in 5% non-fat dry milk, the membranes were incubated overnight at 4 °C with a polyclonal antibodies directed against β-catenin (Sigma, St Louis, MO, USA), phospho-GSK3-β (Ser9), phospho-CREB (Ser133) and acetyl H3 (K18), (all from Cell Signaling Technology, Beverly, MA, USA) rabbit polyclonal anti GFAP (Sigma, STLOUIS, MO, USA) anti IBA (WAKO, Osaka, Japan) anti YM1 (Stemcell, Vancouver, Canada) (primary antibody dilution 1:1000). Monoclonal anti-β-actin and anti β-tubulin antibodies were used as loading controls. Anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences, UK) conjugated to horseradish peroxidase were used for immunodetection. The reactive bands were detected using chemiluminescence (ECL; Amersham Biosciences, Buckinghamshire, UK). Quantitative analysis of reactive bands was performed using the Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Nuclear and cytosol isolation

Nuclei and cytosol were isolated from organotypic hippocampal slices 1 h after the end of the experimental protocols as described in (Gerace et al., 2014). Organotypic slices were homogenized in a glass/glass potter with 500 μl of isolation buffer (TRIS/MOPS 10 mM EGTA/TRIS 1 mM, sucrose 200 mM pH 7.4). Nuclear fraction was obtained after centrifugation at 600 *g* for 10 min and mitochondrial pellet were obtained after centrifugation at 7000*g* for 10 min. Supernatants resulted in cytosolic fraction. Nuclear and cytosolic protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay and processed by Western Blotting.

Statistical analysis

Statistical significance of differences between DCFH2-DA, propidium iodide fluorescence intensities or Western blot optical densities was evaluated by performing analysis of variance (ANOVA) followed by the Tukey's w test for multiple comparisons. Differences were considered significant for $*p < 0.05$, $**p < 0.01$. Graph Pad Prism (Graph Pad Software, San Diego, CA) was used for performing all statistical analysis.

RESULTS

DHPG postconditioning induces neuroprotection by reducing ROS formation and by activating the PI3K/Akt/GSK3β signaling pathway in organotypic hippocampal slices exposed to OGD

As previously reported, organotypic hippocampal slices exposed to DHPG postconditioning (DHPG PostC) showed a significant reduction of neuronal injury induced by 30 min OGD via the activation of PI3K–Akt signaling pathway (Scartabelli et al., 2008). Fig. 1 shows that DHPG PostC is also able to decrease the levels of ROS formation that has been induced by OGD, starting from 6 h after PostC treatment to a maximal effect 24 h later (Fig. 1, panel A and B). This reduction is similar to what obtained by the classical ROS scavenger resveratrol (10 μM), that is also able to induce neuroprotection when used in the same experimental condition of DHPG PostC (Supplementary data, Fig. 1). The decrease in ROS production provoked by DHPG PostC is prevented by the PI3K inhibitor LY294002, indicating that there is a relation between PI3K–Akt pathway activation and ROS formation in the protective mechanisms of PostC (Fig. 1, panel C).

We then examined the hypothesis that the neuroprotection induced by DHPG PostC could be mediated by GSK3β, a downstream target of the above-mentioned signaling pathway. In particular, GSK3β could have an important role in the regulation of β-catenin activation and translocation from cytoplasm to the nucleus, where β-catenin acts as a transcriptional coactivator, activating genes involved in cell survival and neuroprotection. To this aim, we firstly used a

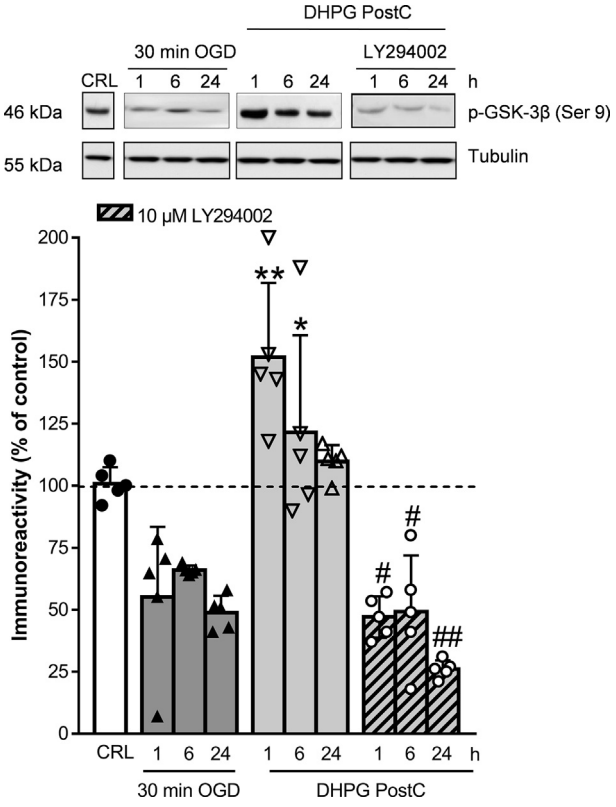

Fig. 2. DHPG PostC induces PI3K–Akt signaling pathway-dependent increase in GSK-3β phosphorylation. 1, 6 or 24 h after treatments Western blot analysis were performed to assess the levels of GSK-3β phosphorylation. *Upper panel*: representative Western blot for phosphorylated GSK-3β (p-GSK-3β). *Lower panel*: quantitative analysis showing that DHPG PostC induced an increase in GSK-3β phosphorylation that was significantly decreased in presence of the Akt inhibitor LY294002. Data are expressed as percentage of GSK-3β phosphorylation in control slices. Bars represent the mean ± SEM of at least five experiments. * $p < 0.05$ and ** $p < 0.01$ vs. CRL. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. 1 h DHPG PostC (ANOVA + Tukey's *w* test).

phospho-specific (Ser9) antibody to measure the levels of GSK3β after exposure to OGD or to DHPG PostC protocol. We observed that OGD induced a decrease in GSK3β phosphorylation at different time point (Fig. 2). On the contrary, pharmacological PostC leads to a transient increase in GSK3β phosphorylation that is maximal 1 h after treatment and returned to basal levels 24 h later (Fig. 2). The PostC-induced increase in GSK3β phosphorylation was reduced by LY294002 (Fig. 2), confirming that the activation of the survival kinase Akt is responsible for GSK3β inactivation.

In addition, we analyzed the cytosolic and nuclear levels of β-catenin, one of the substrates of GSK3β, in order to determine whether it accumulates in the cytoplasm and consequently translocates to the nucleus after DHPG PostC. Our data show that OGD did not affect protein levels neither in cytosol nor in nucleus in organotypic hippocampal slices (Fig. 3). On the contrary, 1 h after DHPG PostC treatment we observed a significant increase of β-catenin expression in both cytosolic and nuclear fractions that is completely reverted to the basal level by Akt inhibitor LY294002 (Fig. 3).

To elucidate whether DHPG PostC could promote neuronal survival through accumulation of nuclear β-catenin, we analyzed the cAMP response element-binding protein (CREB) phosphorylation (Ser133) in our slices after exposure to OGD or to PostC alone or in combination with LY294002. Our results show that PostC induces a significant increase in CREB phosphorylation that is completely prevented by the co-incubation with the Akt inhibitor.

These results propose GSK3β as new target for neuroprotection, therefore, we tested the two GSK3β inhibitors TC-G 24 and LiCl as neuroprotective agents in OGD and also as PostC agents. Our results confirm our hypothesis and show that both GSK3β inhibitors significantly reduce the damage induced by OGD (Fig. 5, panel A and B). Furthermore, slices exposed to TC-G 24 or LiCl for 30 min, 5 min after the toxic OGD insult (PostC protocol) display a significant reduction of CA1 toxicity that is similar to what obtained with DHPG PostC (Fig. 5, panel A and C), confirming and strengthening the crucial role of GSK3β in PostC and neuroprotection.

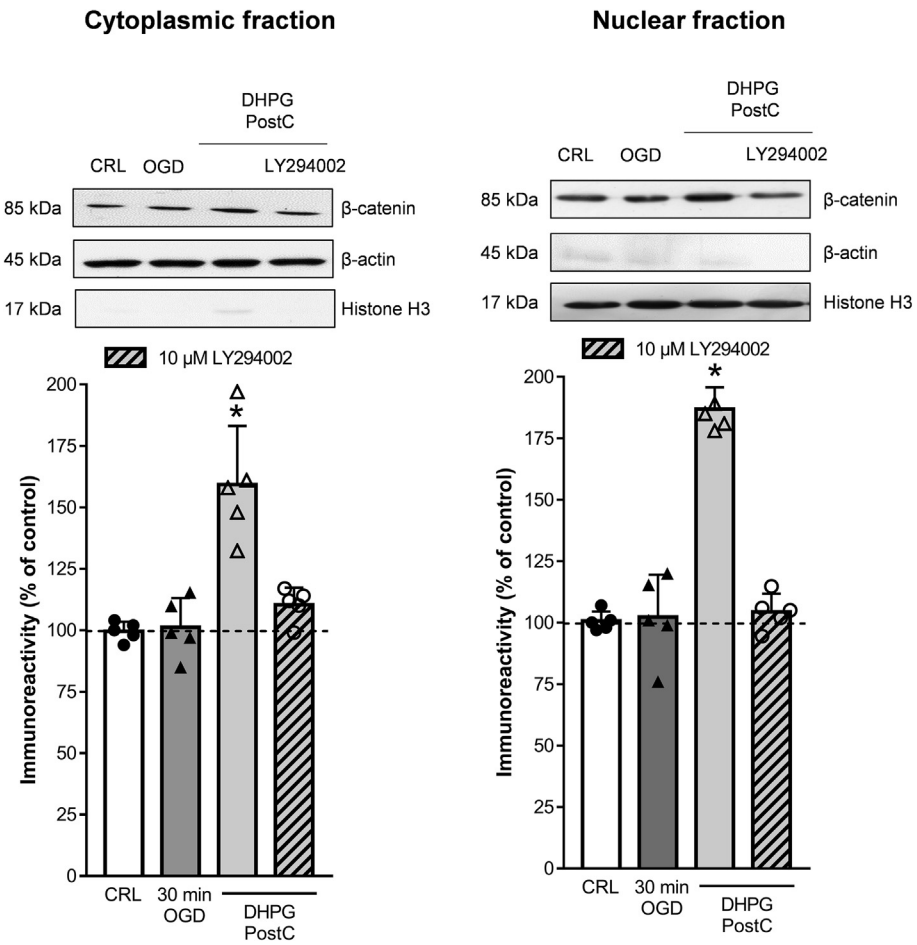

Fig. 3. DHPG PostC is associated with cytosolic and nuclear accumulation of β-catenin. Slices were exposed to 30 min OGD, DHPG PostC and DHPG PostC plus the Akt inhibitor LY294002. The level of β-catenin was determined by Western blot analysis in cytosolic (*left panel*) and nuclear (*right panel*) fractions 1 h after treatments. Representative Western blot for β-catenin (*upper panel*) and quantitative analysis (*lower panel*) of immunoreactive bands showing that DHPG PostC but not OGD induced a significantly increase in β-catenin in both cytosolic and nuclear fractions. This increase is mediated by PI3K–Akt activation and is prevented by the Akt inhibitor LY294002. Data are expressed as percentage of β-catenin in control untreated slices. Data are expressed as percentage of β-catenin in control untreated slices. Bars represent the mean ± SEM of at least five experiments. *$p < 0.05$ vs. CRL (ANOVA + Tukey's *w* test).

DISCUSSION

Ischemic PostC has been shown to be protective against ischemic injury in different organs including liver, intestine and brain (Guo et al., 2011; Liu et al., 2003; Pignataro et al., 2008). The underlying mechanisms were related to multiple factors including regulation of enzyme activity, inhibition of endoplasmic stress, activation of signaling pathways, suppression of oxidative stress and effective regulation of cerebral blood flow to the ischemic area. The present study provides findings that increase our understanding of the signal transduction mechanism of the neuroprotection afforded by pharmacological PostC (10 μM DHPG for 30 min) in a rat hippocampal slice model of cerebral ischemia. In our previous work, we have demonstrated that our PostC protocol increases the phosphorylation of Akt in a transient and mGlu1/mGlu5-dependent manner and that the inhibitors of PI3K and Akt activity

completely abolish the neuroprotection induced by PostC (Scartabelli et al., 2008).

Metabotropic glutamate receptors (mGluRs) of group I (mGluR1 and mGluR5) are specifically localized at pre- and post-synaptic sites in both neurons, glial and microglial cells (Niswender and Conn, 2010; R. et al., 2013). It was previously reported that neurotoxicity induced by OGD is most likely mediated by mGlu1 rather than mGlu5 receptors in organotypic hippocampal slices (Pellegrini-Giampietro, 2003). On the other hand, we observed that DHPG Pre- and PostC induces ischemic tolerance in the same model via the contribution of both mGlu1 and mGlu5 receptors (Werner et al., 2007; Scartabelli et al., 2008). Therefore, a recent paper of our lab have shown that low, subtoxic concentrations of DHPG (1 μM) could also prevent the loss of neurons induced by OGD leading to neuroprotection via mGluR5 (Cavallo et al., 2020). On the contrary, astrocytes were not significantly affected by DHPG (Cavallo et al., 2020) or DHPG PostC (Supplementary data, Fig. 2 panel A). Moreover, in OGD slices treated with DHPG, microglia cells display a morphology typical of reactive microglial (Cavallo et al., 2020), that in DHPG PostC appear to mediate ischemic tolerance by enhancing the protective phenotype M2 without changing the total amount of microglia cells (Supplementary data, Fig. 2 panel B and C).

In this study, we firstly show that DHPG PostC induces neuroprotection against OGD by decreasing the formation of ROS thought Akt activation in organotypic hippocampal slices (Fig. 1 A-C). This reduction is similar to what obtained by the classical ROS scavenger resveratrol, that is also able to induce neuroprotection when used in the same experimental condition of DHPG PostC (Supplementary data, Fig. 1).

The PI3K/Akt signaling pathway plays a central role in regulating cell growth, proliferation and survival under physiologic and pathophysiologic conditions (Cantley, 2002). Gao and colleagues have demonstrated that ischemic PostC enhances the phosphorylation of Akt and that the tolerance induced by PostC in stroke is partly abolished by the inhibition of Akt activity (Gao et al., 2008). It is well known that activation of PI3K is important in the intracellular signaling leading to cardioprotection and neuroprotection by ischemic or pharmacological PostC both in *in vitro* and *in vivo* models (Li and Zuo, 2011; Wang et al., 2010). Many neuroprotectants, including propofol (Wang et al., 2009), estradiol and progesterone (Perez-Alvarez et al., 2015), N-acetyl serotonin (Yoo et al., 2017), thyroid hormone metabolite (Landucci et al., 2019; Laurino et al., 2018), manifest their protective effect through the PI3K/Akt pathway. Downstream targets of PI3K in PostC include the phosphorylation of components of the PI3K/Akt and of GSK3β (Zhou et al., 2011; Qi et al., 2012). Our results show that GSK3β phosphorylation and its subsequent inactivation is necessary for neuroprotection associated with DHPG PostC and the inhibitors of GSK3β are neuroprotective in our OGD model (Fig. 5A and B). The GSK3β inhibitor could also partially protect neurons against glutamate-induced excitotoxicity in primary cultures of rat cerebellar granule neurons (Hu et al., 2013). Moreover, in our model the GSK3β inhibitors TC-G 24 and LiCl are neuroprotective against OGD and could be used as PostC agents (Fig. 5C). Similar results were observed also in *in vivo* models of cerebral ischemia in which lithium improves functional and behavioral outcome in gerbils subjected to two vessels occlusion (Bian et al., 2007) and in rats subjected to MCAO (Taliyan and Ramagiri, 2016). Lithium is able to attenuate OGD injury only in combination with 4-phenylbutirric acid in SK-N-MC neuronal cell cultures (Tung et al., 2015) and does not afford protection in adult hippocampal slices (Rosa et al., 2008).

GSK3β could have a central part in the regulation of β-catenin activation and translocation from cytoplasm to the nucleus, where β-catenin acts as a transcriptional coactivator, activating genes involved in cell survival and neuroprotection. In a recent paper of Tang and collaborators was observed that following global ischemia a variety of Wnt-related proteins, including Wnt and β-catenin (proteins involved in neuroprotection), were significantly reduced, meanwhile, GSK-3β, which promotes negative effects, was significantly increased (Tang et al., 2019). The Wnt/β-catenin signaling pathway

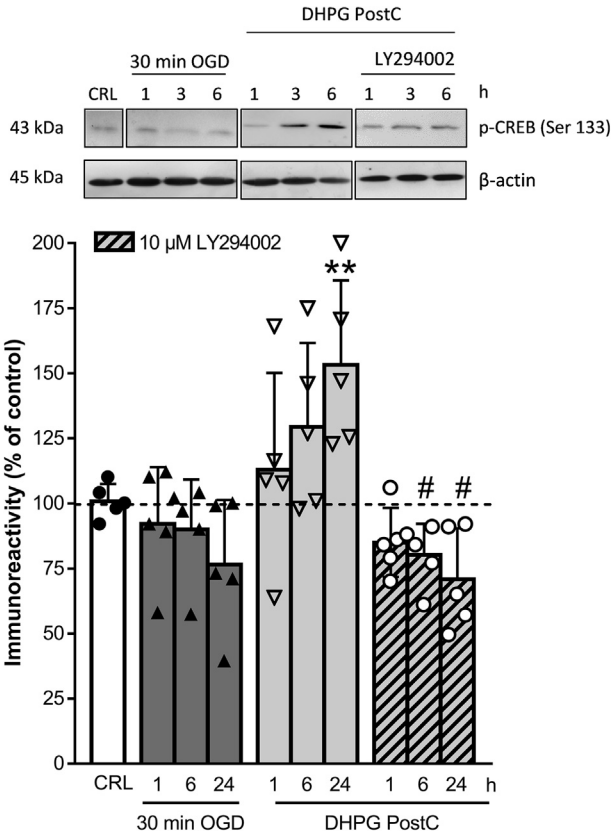

Fig. 4. DHPG PostC induces an increase in CREB phosphorylation. 1, 6 or 24 h after treatments, the levels of CREB phosphorylation was evaluated through Western blot analysis. *Upper panel*: representative image for phosphorylated CREB (p-CREB). *Lower panel*: quantitative analysis of showing that DHPG PostC induced an increase in CREB phosphorylation that was significantly decreased in presence of the Akt inhibitor LY294002. Data are expressed as percentage of CREB phosphorylation in control slices. Bars represent the mean ± SEM of at least five experiments. $^{**}p < 0.01$ vs. CRL, $^{\#}p < 0.05$ vs. 24 h DHPG PostC (ANOVA + Tukey's *w* test).

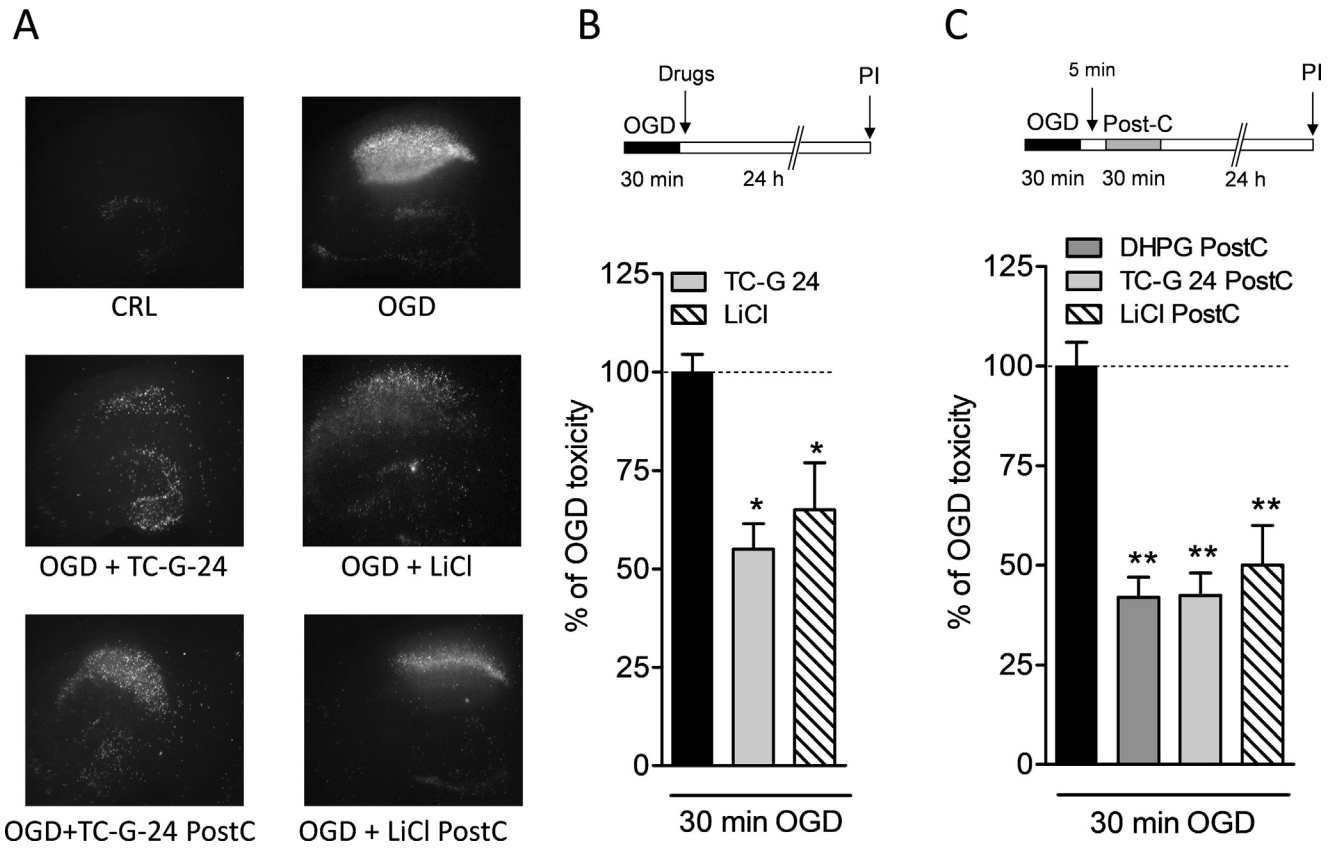

Fig. 5. GS3K inhibitors attenuates neurotoxicity and are involved in the induction of OGD tolerance. **(A)** Control: displaying background levels of PI fluorescence, OGD: displaying an intense PI labeling in CA1 region 24 h later. Slices incubated with TC-G 24 or LiCl after OGD or used as PostC show a reduction of CA1 PI fluorescence **(B)** *Upper panel* showing the experimental protocol. *Lower panel* showing that incubation with the GS3K inhibitors TC-G 24 and LiCl significantly attenuated OGD injury (quantitative analysis). **(C)** *Upper panel* showing the experimental protocol. *Lower panel* showing that slices exposed to DHPG or to TC-G 24 or LiCl PostC significantly attenuated OGD injury (quantitative analysis). Values represent the mean ± SEM of at least three experiments performed in quadruplicate. *$p < 0.05$ and **$p < 0.01$ vs. 30 min OGD alone (ANOVA + Tukey's *w* test).

was downregulating by hypoxia–ischemia in H9C2 cells (Tao et al., 2016) and its dysfunction in temporal lobe epilepsy is responsible of structural and functional abnormalities, while restoring Wnt pathway leads to neuroprotective effects (Huang et al., 2015). Similarly, the neuroprotective effects of isoflurane PostC is mediated by Wnt/β-catenin signaling in MCAO models of cerebral ischemia in rats (Zhang et al., 2019).

In our DHPG PostC model, we also observed accumulation of β-catenin in the cytoplasm and translocation from the cytosol to nucleus (Fig. 3), which were dependent on PI3K/Akt/GSK3β activity.

The activation of the Wnt pathway through different ligands, such as lithium, induced an increase in intracellular calcium levels that trigger a sequence of events that include CREB phosphorylation and transcription of CREB-dependent genes that promote survival (Arrázola et al., 2009). In accordance, DHPG PostC induced an increase in CREB phosphorylation in a time dependent manner and Akt inhibitors reverted this effect (Fig. 4).

In conclusion, our results show that PostC induced neuroprotection by the activation of PI3K/Akt/GSK3β pro-survival pathway. In addition, GSK3β is not only a downstream target of this pathway, but is also involved in the Wnt pathway and its inactivation leads to β-catenin accumulation and translocation from the cytosol into the nucleus, that ultimately leading to CREB phosphorylation and transcription of CREB-dependent genes that promote survival.

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APPENDIX A. SUPPLEMENTARY DATA

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