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Cyclosporine-A mimicked the ischemic pre- and postconditioning-mediated cardioprotection in hypertensive rats: Role of PKCε

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ABSTRACT

Our aim was to assess the action of cyclosporine-A (CsA) against reperfusion injury in spontaneously hypertensive rats (SHR) compared to the effects of ischemic pre- (IP) and postconditioning (IPC), examining the role played by PKCε. Isolated hearts were submitted to the following protocols: IC: 45 min global ischemia (GI) and 1 h reperfusion (R); IP: a cycle of 5 min GI and 10 min of R prior to 45 min-GI; and IPC: three cycles of 30 s-GI/30 s-R at the start of R. Other hearts of the IC, IP and IPC groups received CsA (mitochondrial permeability transition pore inhibitor) or chelerythrine (Che, non-selective PKC inhibitor). Infarct size (IS) was assessed. TBARS and reduced glutathione (GSH) content — as parameters of oxidative damage, the expression of P-Akt, P-GSK-3β, P-PKCε and cytochrome c (Cyc) release — as an index of mitochondrial permeability and the response of isolated mitochondria to Ca^{2+} were also measured. IS similarly decreased in preconditioned, postconditioned and CsA treated heart showing the highest values in the combinations IP + CsA and IPC + CsA. TBARS decreased and GSH was partially preserved after all interventions. The content of P-Akt, P-GSK-3β and P-PKCε increased in cytosol and decreased in mitochondria after IP and IPC. In CsA treated hearts these enzymes increased in both fractions reaching the highest values. Cyc release was attenuated and the response of mitochondria to Ca^{2+} was improved by the interventions. The beneficial effects of IP and IPC were annulled when PKC was inhibited with Che. A PKCε/VDAC association was also detected. These data show that, in SHR, the CsA treatment mimicked and reinforced the cardioprotective action afforded by IP and IPC in which PKCε-mediated attenuation of mitochondrial permeability appears as the main mechanism involved.

1. Introduction

The left ventricular hypertrophy consequent to chronically elevated blood pressure has been frequently associated with postischemic contractile dysfunction (Friehs and del Nido, 2003). It was previously shown in stroke-prone spontaneously hypertensive rats (SHR-SPs) (Chen et al., 2000; Yano et al., 2011) and recently in spontaneously hypertensive rats (SHR) (Fantinelli et al., 2013) that hypertrophy aggravates the irreversible reperfusion injury. It was also demonstrated in our and in other laboratories that ischemic pre- (IP) and postconditioning (IPC) decrease myocardial dysfunction (Fantinelli et al., 2013; Fantinelli and Mosca, 2007) and limit the infarct size in hypertrophied hearts (Ferdinandy et al., 2007; Speechly-Dick et al., 1994).

It is well established that hypertension induces oxidative stress, but major sources of reactive oxygen species (ROS) are not absolutely certain, raising the possibility that NADPH oxidase, nitric oxide synthase, lipoxygenases, cyclo-oxygenases, xanthine oxidase, and cytochrome P450 enzymes, and the mitochondrial respiratory chain may be important ROS producers (Puddu et al., 2008; Lesnefsky et al., 2001). ROS are implicated in the mitochondrial permeability transition pore (mPTP) opening which plays a crucial role in the ischemia–reperfusion-induced cell death (Halestrap, 2009; Ong et al., 2015). Regardless of all controversies in resolving the molecular enigma of mPTP, a general consensus

Abbreviations: CsA, cyclosporine A; SHR, spontaneously hypertensive rats; IP, ischemic preconditioning; IPC, ischemic postconditioning; PKCε, protein kinase C ε; Che, chelerythrine; TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; Akt, Serine/threonine-specific protein kinase; Akt, Serine/threonine-specific protein kinase; Cyc, cytochrome c; VDAC, voltage-dependent anion channels; mPTP, mitochondrial permeability transition pore; CyD, cyclophilin D; TTC, triphenyltetrazolium chloride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPCR, G protein-coupled receptors; GP, G-protein; CN, calcineurin; Akt, serine/threonine-specific protein kinase; ROS, radical oxygen species; ANT, adenine nucleotide translocator.

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exists on the role of cyclophilin D (CyD) as a regulator of mPTP (Ong et al., 2015). Indeed, the immunosuppressive agent, cyclosporine A (CsA) was effective in reducing infarct size in patients (Hausenloy et al., 2012; Piot et al., 2008) and in experimental studies performed in normotensive animals (Javadov and Kuznetsov, 2013; Nakagawa et al., 2005; Mewton et al., 2010). However, its effects in hypertensive animals have not yet been reported.

On the other hand, it was reported that PKC and specifically the ε isoform (PKCε) have an important role in IP triggering process and is a potential mediator of IPC (Budas and Mochly-Rosen, 2007; Baines et al., 2002; Yoshida et al., 1997; Inagaki et al., 2003) in normotensive animals. According to a previous paper (Johnsen et al., 2005) the expression of PKCε in hypertensive is higher – approximately 3 fold – than normotensive rats. In a previous work performed in our laboratory (Fantinelli and Mosca, 2007) the postischemic myocardial dysfunction of hearts from SHR was severely affected when PKC was inhibited indicating that this kinase plays a protective role against myocardial stunning. If PKC is also involved in cell death caused by a more prolonged ischemia has not been properly clarified.

Therefore, our objective was to compare the effects of CsA treatment with those obtained by IP and IPC on infarct size and oxidative stress in isolated hearts from SHR examining the role played by PKCε.

2. Materials and methods

An expanded 'Methods' section is available in Online Data Supplements.

2.1. Isolated rat heart

All procedures followed during this investigation were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, University of La Plata following the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research, National Research Council, Washington, D.C., National Academy Press, 2010 and/or European Union Directive for Animal Experiments 2010/63/UE.

Experiments were conducted in 5-month-old SHR, which were originally derived from Charles River Breeding Farms, Wilmington, Mass. Systolic blood pressure (SBP) was measured weekly using the methods indicated in Supplementary material online. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt). The heart was rapidly excised and perfused by the non-recirculating Langendorff technique and it was paced at 280 ± 10 beats/min.

2.2. Experimental protocols

After 30 min of stabilization, hearts from SHR were assigned to the following experimental protocols (Fig 1): Non-ischemic control hearts (NIC; n = 6): Hearts were perfused for 135 min without any treatment and ischemic control hearts (IC; n = 8): Hearts were subjected to 45 min of normothermic global ischemia followed by 1 h of reperfusion. Global ischemia was induced by stopping the perfusate inflow line and the heart was placed in a saline bath held at 37 °C; ischemic preconditioning (IP, n = 8): One cycle of 5 min of ischemia and 10 min of reperfusion was previously applied to the 45-min ischemic period followed by 1-hour reperfusion; and ischemic postconditioning (IPC, n = 8): Three cycles of 30 s of ischemia and 30 s of reperfusion was applied early during reperfusion.

Chelerythrine (Che) treatment: Hearts were treated with 1 μM Che (PKC inhibitor), 10 min before 45-min ischemia (IC + Che, n = 4; IPC + Che, n = 5) or 5-min ischemia (IP + Che, n = 6).

Cyclosporine A (CsA) treatment: Hearts were treated with 0.5 μM CsA (mPTP inhibitor), 10 min before 45-min ischemia (IC + CsA, n = 4; IPC + CsA, n = 4) or 5-min ischemia (IP + CsA, n = 5).

In other hearts (n = 3) CsA was added to non-ischemic control hearts. Separated groups of hearts subjected to the same protocols (n = 6 for each one) were used for biochemical determinations. Additional hearts submitted to the different protocols (n = 4 for each one) were used for mitochondria isolation.

2.3. Infarct size determination

Infarct size was assessed by the widely validated triphenyltetrazolium chloride (TTC) staining technique and expressed as a percentage of area at risk.

2.4. Preparation of tissue homogenate

At the end of reperfusion a portion of left ventricle (LV) was homogenized in 5 volume of 25 mmol/L PO_4KH_2–140 mmol/L ClK at pH = 7.4

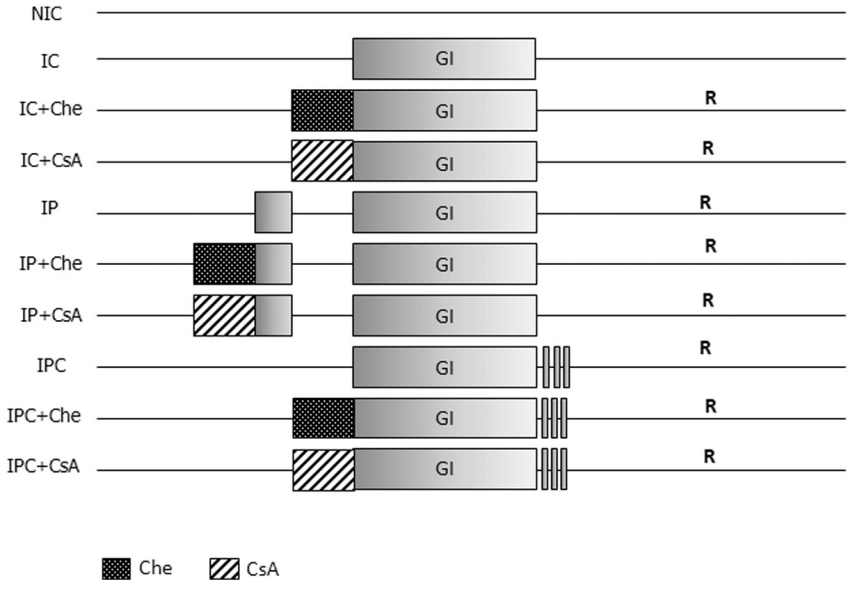

Fig. 1. Scheme of the experimental protocols. NIC: non-ischemic control; IC: ischemic control; IC + Che: ischemic control in the presence of chelerythrine (Che) inhibitor of PKC; IC + CsA: ischemic control in the presence of cyclosporine A (CsA), inhibitor of cyclophilin D; IP: ischemic preconditioning; IP + Che: ischemic preconditioning plus Che; IP + CsA: ischemic preconditioning plus CsA; IPC: ischemic postconditioning; IPC + Che: ischemic postconditioning plus Che; IPC + CsA: ischemic postconditioning plus CsA.

Table 1
Values of body weight (BW, g), heart weight (HW, mg), hypertrophy index (HI) and systolic blood pressure (SBP, mm Hg) of all experimental groups.

Group	BW (g)	HW (mg)	HI	SBP (mm Hg)
NIC	336 ± 12	1500 ± 40	4.5 ± 0.1	216 ± 5
IC	328 ± 10	1530 ± 70	4.7 ± 0.2	204 ± 8
IC + Che	328 ± 11	1500 ± 30	4.6 ± 0.1	218 ± 7
IC + CsA	329 ± 13	1490 ± 90	4.8 ± 0.2	210 ± 4
IP	329 ± 13	1430 ± 40	4.4 ± 0.2	208 ± 8
IP + Che	331 ± 17	1550 ± 70	4.7 ± 0.5	203 ± 4
IP + CsA	320 ± 19	1540 ± 60	4.8 ± 0.2	209 ± 4
IPC	325 ± 13	1520 ± 70	4.7 ± 0.2	213 ± 4
IPC + Che	326 ± 14	1200 ± 70	4.3 ± 0.3	219 ± 6
IPC + CsA	330 ± 12	1570 ± 50	4.7 ± 0.2	214 ± 5

with a Polytron homogenizer. Aliquots of homogenate were used to assess reduced glutathione (GSH) content and thiobarbituric reactive substance (TBARS) concentration.

2.5. Immunoblotting

Other portion of LV was homogenized and mitochondrial and cytosolic fractions were isolated by differential centrifugation. Both fractions were resolved on SDS-PAGE, transferred to PVDF membranes and probed with antibodies anti-P-PKCε, anti-P-GSK-3β, anti-P-Akt, and anti-cytochrome c. GAPDH and VDAC were used for control in the cytosolic and mitochondrial fractions, respectively.

2.6. Coimmunoprecipitation

Supernatants obtained from the first homogenization of the hearts were applied to A Sepharose protein. After centrifugation lysates were incubated with a rabbit polyclonal anti-VDAC antibody and A Sepharose protein. Samples were electrophoresed and immunoblots were probed with an anti-P-PKCε antibody or anti-VDAC antibody.

2.7. Isolation of mitochondria

Hearts were immediately removed from rats, and mitochondria from left ventricle (LV) were isolated by differential centrifugation.

2.8. Ca^{2+}-induced mitochondrial swelling

After 5-min preincubation, 0.3 mg/mL of mitochondrial suspension energized with 6 mmol/L succinate were induced to swell by the addition of $CaCl_2$ 200 μmol/L. These changes are observed as decreases of light scattering at excitation and emission wavelengths of 520 nm and calculated taking the difference of scattered light between before and after the addition of $CaCl_2$.

2.9. Statistical analysis

Data are presented as mean ± SE and repeated measures of two-way analysis of variance (ANOVA) with Newman–Keuls test were used for multiple comparisons among groups. A p value < 0.05 was considered significant.

3. Results

Table 1 shows that the used animals for the different experimental groups presented similar high values of body and heart weight, hypertrophy index and SBP.

3.1. Infarct size

The addition of CsA did not modify the infarct size observed in non-ischemic control hearts (1.6 ± 0.4%). As shown in Fig. 2, infarct size, expressed as percentage of area at risk (AR) of ischemic control (IC) hearts was significantly reduced by IP and IPC (34 ± 1% and 33 ± 1% vs. 51 ± 4%). The inhibition of PKC with chelerythrine (Che) before ischemia or at the beginning of reperfusion abolished the protection

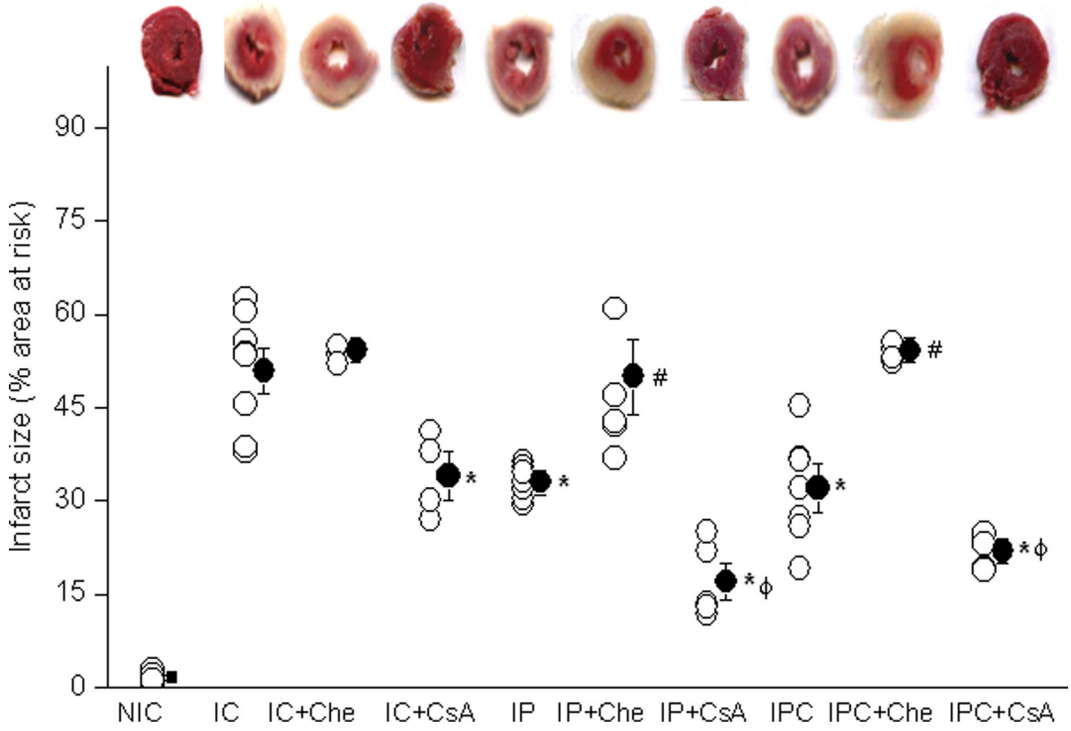

Fig. 2. Infarct size (IS), expressed as a percentage of risk area, in non-ischemic control (NIC), ischemic control (IC), IC + Che, IC + CsA, preconditioned (IP), IP + Che, IP + CsA, postconditioned (IPC), IPC + Che and IPC + CsA. Observe that all interventions decreased the IS detected in IC hearts. Che abolished the protection achieved by IP and IPC. CsA decreased the IS of IC hearts but its administration to IP or IPC hearts did not confer additional protection. *p < 0.05 vs. IC; #p < 0.05 vs. IP or IPC; ϕp < 0.05 vs. IP or IPC (for CsA).

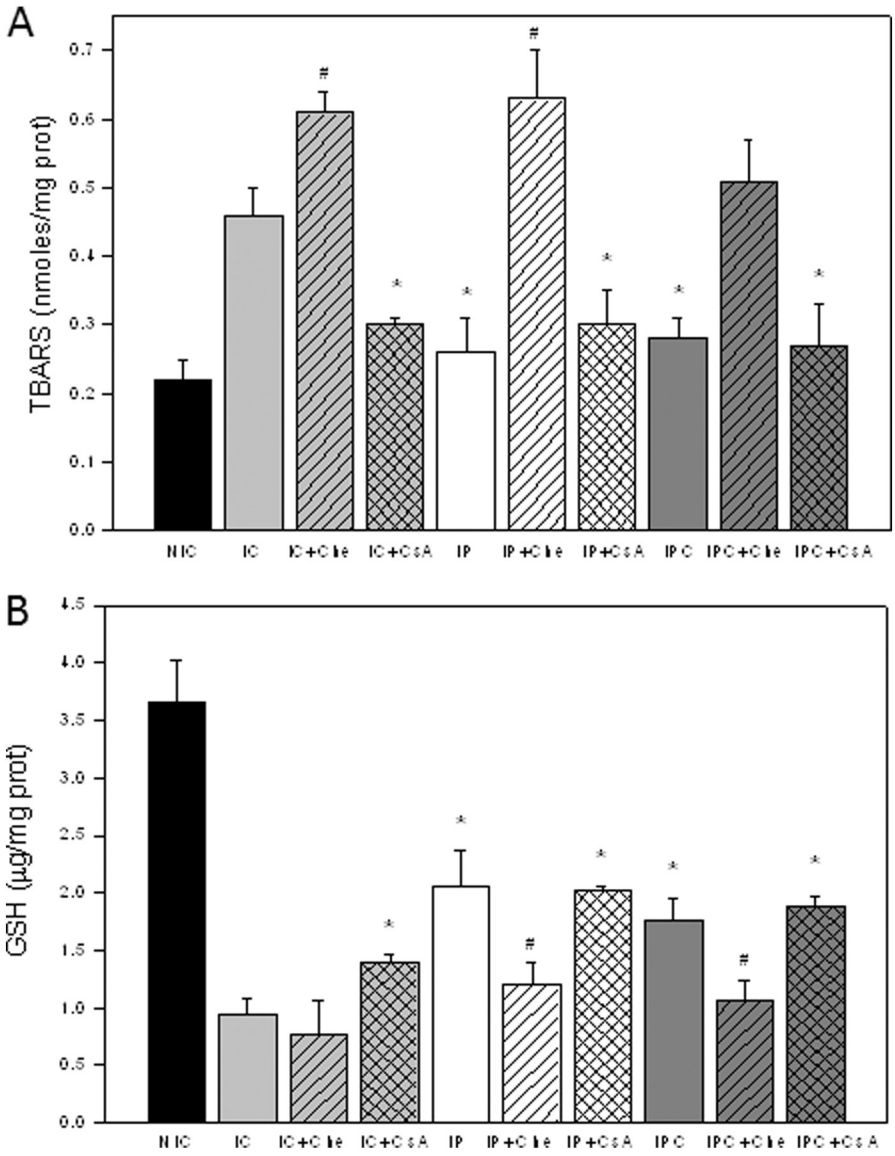

Fig. 3. Thiobarbituric acid reactive substance concentration (TBARS, nmol/mg protein, A panel), reduced glutathione content (GSH, μg/mg protein, B panel) in all experimental groups. After ischemia and reperfusion (IC hearts) TBARS increased and GSH levels diminished. The preconditioned (IP), postconditioned (IPC) and CsA treated hearts attenuated these changes, partially preserving the GSH content and diminishing TBARS content. Che reversed the changes produced by IP and IPC. *$p < 0.05$ vs. IC; #$p < 0.05$ vs. IP or IPC.

afforded by those interventions. The treatment of IC hearts with cyclosporine A (CsA) decreased the cell death (IS = 34 ± 4%) at a level similar to that obtained with IP or IPC. However, the aggregate of CsA to IP and IPC conferred additional cardioprotection.

3.2. Thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH)

Lipid peroxidation – assessed by TBARS – increased in IC hearts and was similarly attenuated by IP, IPC, CsA, IP + CsA and IPC + CsA. Che treatment returned the TBARS to similar or higher values to those observed in the IC group (Fig 3, A panel). GSH levels diminished in IC compared to NIC hearts and it was partially but significantly preserved by IP, IPC, CsA, IP + CsA and IPC + CsA (Fig. 3, B panel). The PKC blockade annulled the beneficial effects of the interventions being the GSH levels significantly lesser than those of the IP and IPC groups and similar to those of the IC group.

3.3. Phospho-GSK-3β (P-GSK-3β), phospho-Akt (P-Akt) and phospho-PKCε (P-PKCε) expression in cytosolic and mitochondrial fractions

In cytosolic fraction the content of P-GSK-3β, P-Akt and P-PKCε decreased in IC compared to NIC hearts (Fig. 4). IP and IPC increased the expression of the phosphorylated forms of those kinases. The status of P-GSK-3β coincided with that of P-Akt, suggesting that Akt controls the activity of GSK-3β. PKC inhibition with Che did not modify the level of P-GSK-3β and P-Akt but significantly attenuated the P-PKCε level. CsA treatment increased the level of phosphorylated forms of the three kinases until values are similar to those obtained after IP and IPC. When CsA was added to IP and IPC protocols (IP + CsA and IPC + CsA groups) the expression of P-GSK-3β, P-Akt and P-PKCε – except in the IPC + CsA group – increased even further. When the mitochondrial fraction was analyzed the expression of P-GSK-3β and P-Akt was a mirror image of that found in the cytosol (Fig. 5). Thus, IC hearts showed an increase of the content of the phosphorylated forms of these kinases whereas in IP and IPC hearts decreased until levels were

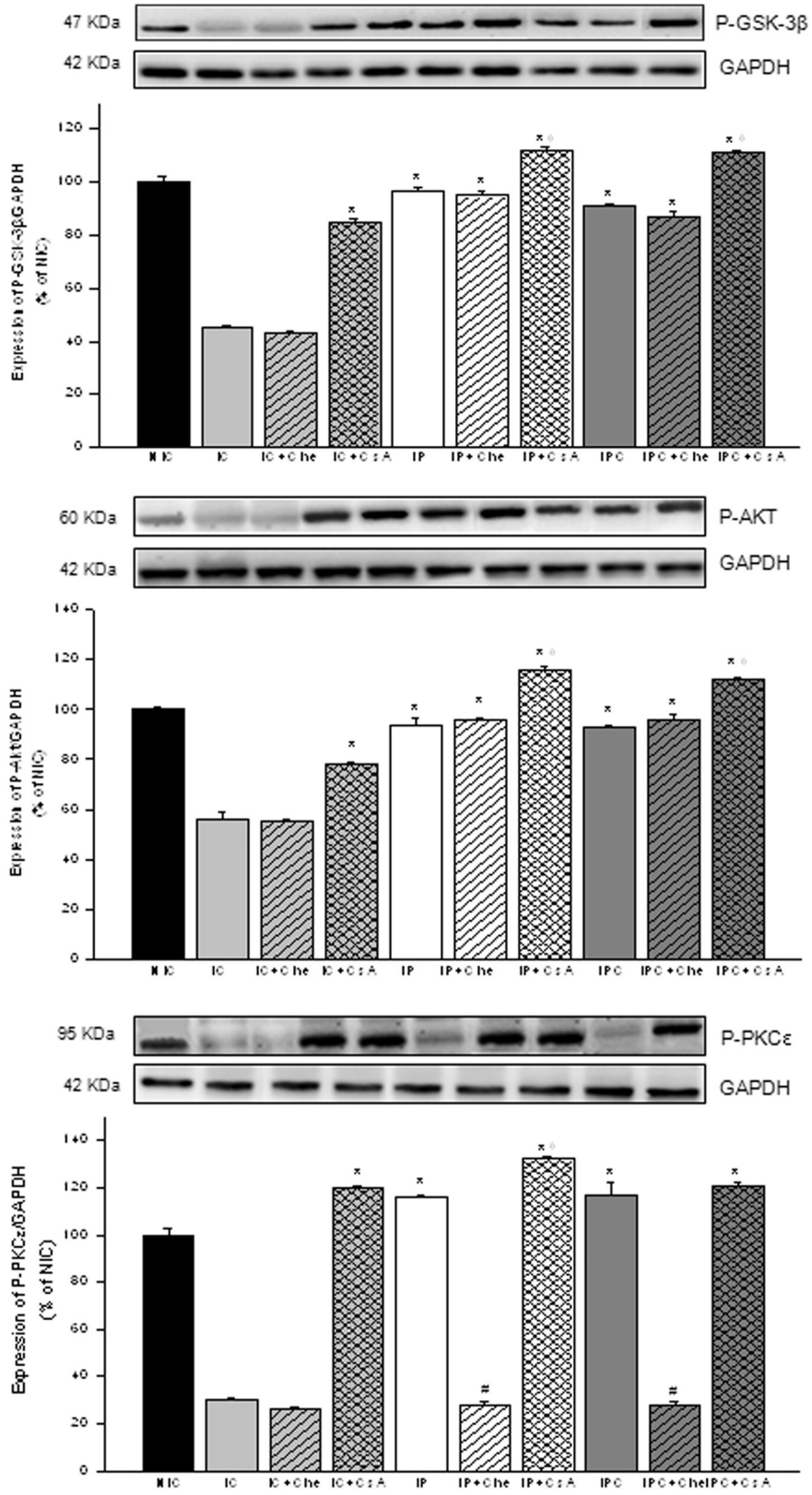

Fig. 4. Representative immunoblots of phosphorylated forms and summary of densitometry data of P-GSK-3β, P-Akt and P-PKCε level in cytosolic fraction in non-ischemic control (NIC), ischemic control (IC), IC + Che, IC + CsA, preconditioned (IP), IP + Che, IP + CsA, postconditioned (IPC), IPC + Che and IPC + CsA groups. Immunoblots of IC hearts showed less levels of cytosolic P-GSK-3β/GAPDH, P-Akt/GAPDH and P-PKCε/GAPDH ratios and they were significantly increased by all the interventions detecting the highest content when CsA was applied. Che abolished the effects of IP and IPC. *$p < 0.05$ vs. IC; #$p < 0.05$ vs. IP or IPC (for Che); ϕ$p < 0.05$ vs. IP or IPC (for CsA).

lower than those observed in the NIC group. These values were not modified by Che. The CsA treatment did not change the levels of P-GSK-3β and P-Akt of IC hearts but when it was added to IP or IPC the expression of these kinases decreased. The content of P-PKCε in IC hearts was not modified compared to the NIC group but greatly increased when CsA was added. In the IP and IPC groups, the content of

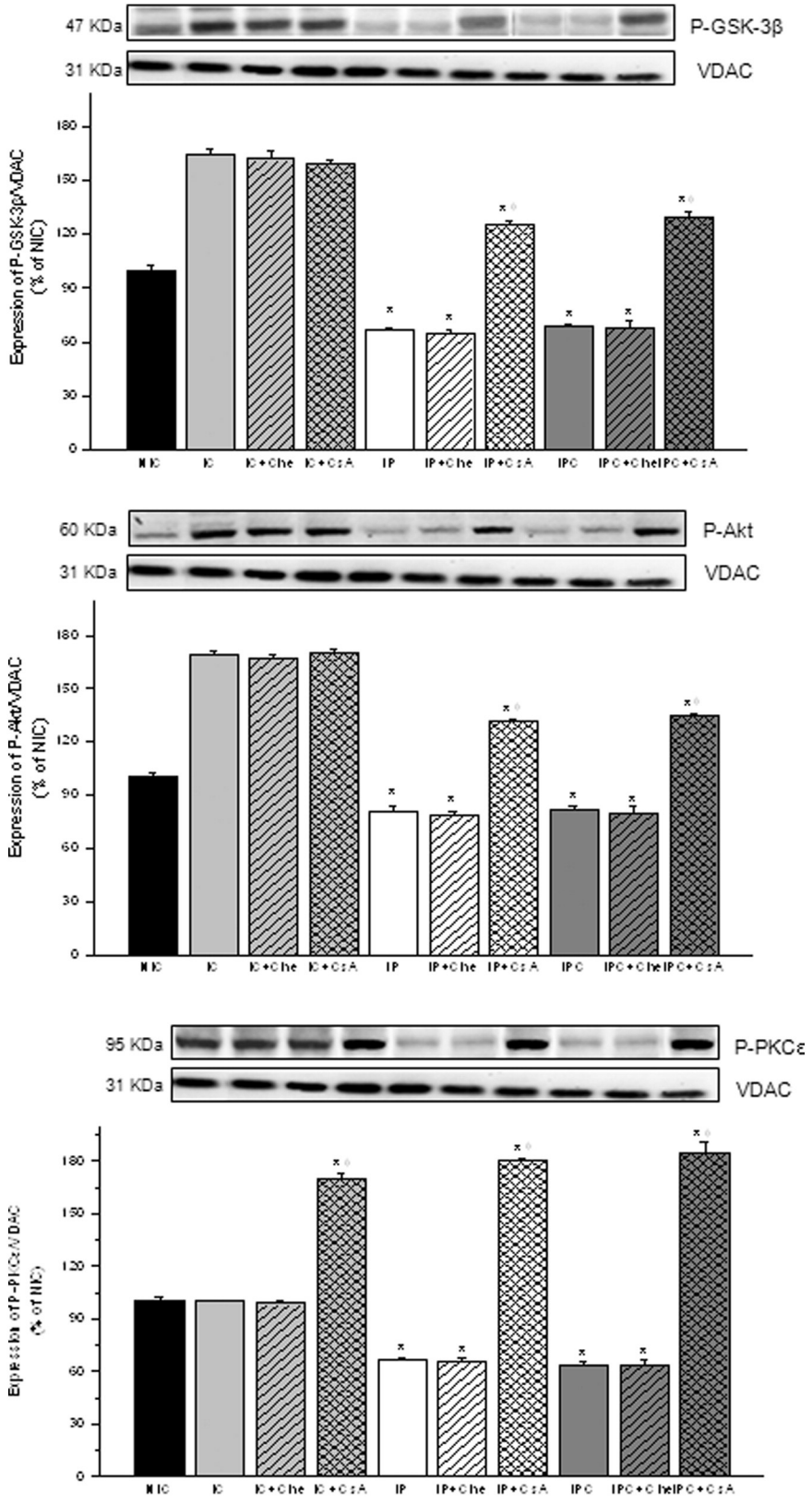

Fig. 5. Representative immunoblots of phosphorylated forms and summary of densitometry data of P-GSK-3β, P-Akt and P-PKCε level in mitochondrial fraction in non-ischemic control (NIC), ischemic control (IC), IC + Che, IC + CsA, preconditioned (IP), IP + Che, IP + CsA, postconditioned (IPC), IPC + Che and IPC + CsA groups. IC hearts showed high levels of P-GSK-3β/VDAC and P-Akt/VDAC ratios and the P-PKCε/VDAC ratio did not change. IP and IPC significantly decreased the content of all kinases whereas CsA increased it reaching P-PKCε higher values in comparison to IC. Che annulled the effects of IP and IPC. *$p < 0.05$ vs. IC; #$p < 0.05$ vs IP or IPC (for Che); ϕ$p < 0.05$ vs. IP or IPC (for CsA).

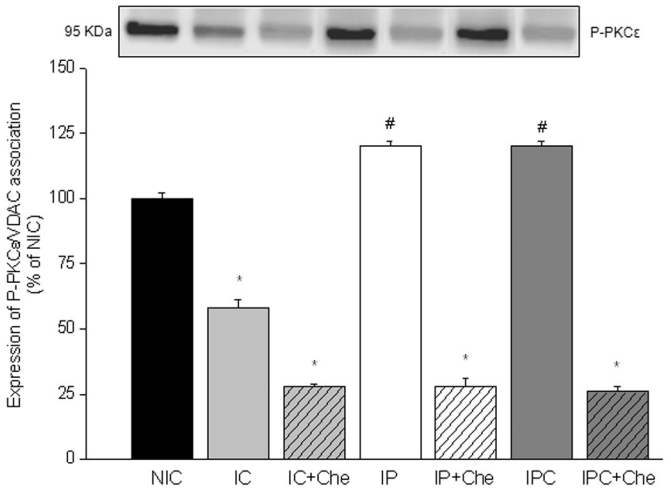

Fig. 6. Representative immunoprecipitated and summary data of P-PKCε/VDAC association in cytosolic fraction from non-ischemic control (NIC), ischemic control (IC), preconditioned (IP), postconditioned (IPC) and treated with Che. These data show that the P-PKCε/VDAC association was minimal in IC hearts and maximal in IP and IPC hearts. This association decreased in the presence of Che. *$p < 0.05$ vs. IC; #$p < 0.05$ vs. IP or IPC.

P-PKCε significantly decreased and was not changed when PKC was inhibited with Che.

3.4. P-PKCε and VDAC physical association

The coimmunoprecipitation shows that the P-PKCε/VDAC association decreased in ischemic control hearts, increased in pre- and postconditioned hearts and was significantly diminished by Che treatment (Fig. 6).

3.5. Cytosolic cytochrome c (cyc) level

In cytosolic fraction obtained from hearts belonging to the IC group the cyc level increased and it was reduced in IP, IPC and CsA treated hearts. PKC inhibition with Che abolished the beneficial effects of IP and IPC returning to values similar to that observed in IC hearts (Fig. 7, A panel). In mitochondrial fraction the changes were opposite to those found in cytosol fraction. Thus, the cyc content decreased in IC hearts and increased in the IP, IPC and CsA groups. The Che treatment annulled the effects of the interventions (Fig. 7, B panel). The addition of CsA to pre- and postconditioned hearts did not alter the level of cyc in both fractions.

3.6. Ca^{2+}-induced mitochondrial swelling

Fig 8 shows typical traces of swelling experiments (A panel) and the mean values of light scattering decrease (LSD, B panel) produced by the addition of 200 μmol/L Ca^{2+} to a mitochondrial suspension from all experimental groups. Mitochondria isolated from IC hearts showed a lesser sensitivity to Ca^{2+} compared to those obtained from the NIC group (0.06 ± 0.01 vs. 0.74 ± 0.07 a.u.). IP, IPC and CsA treatment improved the response of mPTP to Ca^{2+} showing a greater change of fluorescence after Ca^{2+} addition. The PKC inhibition with Che abolished the beneficial changes observed after IP and IPC. The addition of CsA to IP and IPC maintained the Ca^{2+}-induced mitochondrial swelling observed in pre- and postconditioned hearts. These results suggest that the interventions and or CsA treatment are able to diminish the proportion of permeabilized and/or damaged mitochondria produced by ischemia and reperfusion.

4. Discussion

The present study demonstrates that CsA treatment – similarly to IP and IPC – decreases the infarct size and oxidative damage and improves the response of mPTP to Ca^{2+} in hearts from SHR submitted to ischemia and reperfusion. Another important finding is that cytosolic PKCε appears playing a crucial role in the cardioprotection achieved by IP and IPC in hypertensive rats.

It has been previously reported that CsA treatment exerts cardioprotection against ischemia–reperfusion injury in different experimental models using normotensive animals (Hausenloy et al., 2012; Piot et al., 2008; Griffiths and Halestrap, 1993; Gerczuk and Kloner, 2012; Onishi et al., 2012). Also, it was showed (Lim et al., 2011) that mice lacking CypD have less mortality and smaller infarct size, and undergo less adverse left ventricular remodeling. In this study we demonstrated that CsA treatment, similarly to IP or IPC, attenuates the post-ischemic alterations in hypertensive rats. However, when CsA was added to pre- or post-conditioned hearts a greater limitation of infarct size than single interventions was observed.

What mechanisms are implicated? Most of the research performed in normotensive animals support the notion that phosphorylation at Ser9/inhibition of GSK-3β and PI3K/Akt is required for the IP and IPC-mediated cardioprotection (Juhaszova et al., 2009; Zhu et al., 2006). In a recent paper, we presented evidence about the important role played by those kinases in the beneficial effects of IP and IPC in hypertensive rats (González Arbeláez et al., 2013).

The involvement of PKC, specifically ε isoform in the cardioprotection against reperfusion injury has been previously reported (Budas and Mochly-Rosen, 2007; Baines et al., 2002; Yoshida et al., 1997; Inagaki et al., 2003). It is also established that PKCε translocation to multiple intracellular sites is necessary to exert a protective effect (Disatnik et al., 1995; Uecker et al., 2003; Yonekawa and Akita, 2008). On the other hand, PKC isoforms – including PKCε – are activated during compensatory hypertrophy (Takeishi et al., 2000) and their contribution to ameliorate myocardial stunning in SHR was previously reported by us (Fantinelli et al., 2013). In the present experimental design, when a more prolonged ischemic period was applied to isolated hearts, the reduction of cell death detected in pre- and post-conditioned hearts was also annulled when PKC was inhibited. These data clearly define the important role of this kinase in the infarct size limitation afforded by the interventions. An increase in the cytosolic – which was diminished by Che – and a decrease in the mitochondrial P-PKCε content – not affected by Che – after IP and IPC was detected. These data indicate that cytosol PKCε activation appears to be sufficient to provide protection and suggest that translocation of that kinase – at least to the mitochondria – would not be essential.

Simultaneously, an increase of P-GSK-3β and P-Akt in cytosolic fraction was observed after the interventions but these levels were not modified by Che. In CsA treated hearts, similarly to IP and IPC, the content of P-GSK-3β, P-Akt and P-PKCε in cytosol fraction increased, showing the combinations (IP + CsA and IPC + CsA) the highest values. This effect on kinase content could be related to the greater reduction of infarct size obtained in these groups. In mitochondrial fraction CsA treatment did not modify the expression of P-GSK-3β and P-Akt but increased the P-PKCε. The combinations showed the same tendency than CsA alone. These effects could be explained by CsA-dependent but IP and IPC-independent pathways. In this regard, it was previously demonstrated that CsA is able to inhibit Ca^{2+}-calmodulin-dependent protein phosphatase calcineurin which is implicated in hypertrophic signals (Sussman et al., 1998) and mitochondrial fragmentation (Cereghetti et al., 2008).

Therefore, P-Akt/P-GSK-3β and cytosolic P-PKCε-mediated pathways are contributing to CsA, IP and IPC-mediated limitation of infarct size.

At this point we should remember the harmful role played by reactive oxygen species (ROS) and aldehyde accumulation occurring during reperfusion (Downey, 1990). IC hearts showed an increase of TBARS –

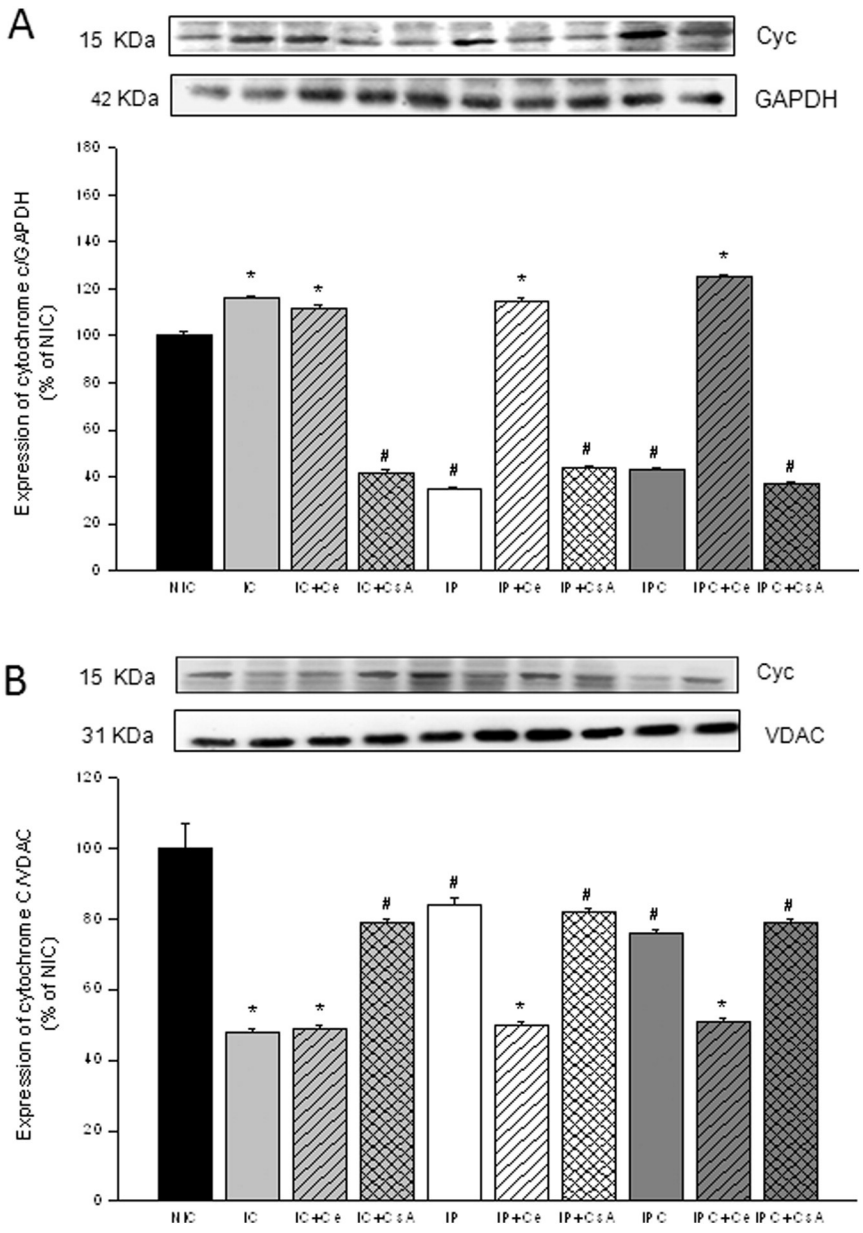

Fig. 7. The cytochrome c (Cyc) content in cytosolic (A panel) and mitochondrial (B panel) fractions of all experimental groups. Note that Cyc content of ischemic control hearts (IC) increased in cytosol and decreased in mitochondria whereas opposite changes were observed after interventions (IC + CsA, IP, IP + CsA, IPC, and IPC + CsA groups). Che returned the Cyc release to that obtained in IC hearts. *$p < 0.05$ vs. NIC; #$p < 0.05$ vs. IC.

an index of lipid peroxidation – simultaneously with a depletion of GSH antioxidant system. That is, the hypertrophied hearts exhibited a significant imbalance between ROS production and antioxidant defense mechanisms – oxidative stress – after 45 min of ischemia and 60 min of reperfusion. In IP, IPC and CsA treated hearts the lipoperoxidation decreased and GSH content was partially restored. In other words the interventions attenuate the oxidative damage. It has previously been demonstrated that oxidative stress increases mPTP activity then having a long-lasting opening which favors the ROS production and cytochrome c release (Clarke et al., 2008). Thus, the reduction of mPTP opening would lead to a diminution of both factors (ROS and cyc). In our study, isolated mitochondria of intervened hearts showed an improvement of mPTP response to Ca^{2+} and a decrease of cytochrome c release indicating a partial preservation of mitochondrial integrity. Although we did not measure ROS levels, a normalization of its production in preconditioned, postconditioned and CsA-treated hearts has been reported (Zhao et al., 2003; Aldakkak et al., 2013). Also, we detected that PKC inhibition with Che annulled these beneficial effects afforded by IP and IPC indicating that PKC is implicated in the understatement of mitochondrial permeability.

Which is/are the target/s of PKC for cardioprotection? The mechanisms include regulation of sarcolemmal and/or mitochondrial KATP channels, regulation of gap-junction permeability through phosphorylation of connexion, interaction with mitochondrial proteins such as cytochrome C oxidase subunit IV, and direct or indirect regulation of mPTP opening (Costa and Garlid, 2008; Garg and Hu, 2007; Bao et al., 2007; Ogbi and Johnson, 2006; Korge et al., 2002). Results from coimmunoprecipitation suggest that the preservation of mitochondrial integrity appears dependent of P-PKCε/VDAC interaction. Thereby, PKCε could be contributing to a decrease of ROS production and/or release in intervened hearts. Recent studies propose that activation via PKCε of aldehyde dehydrogenase 2 (ALDH2) – enzyme responsible for the metabolization of toxic reactive aldehydes such as 4 aldehyde 4-hydroxy-2-nonenal (4-HNE) – is cytoprotective (Gomes et al., 2014). The exacerbation of oxidative damage in the presence of Che suggests that PKC-mediated ALDH2

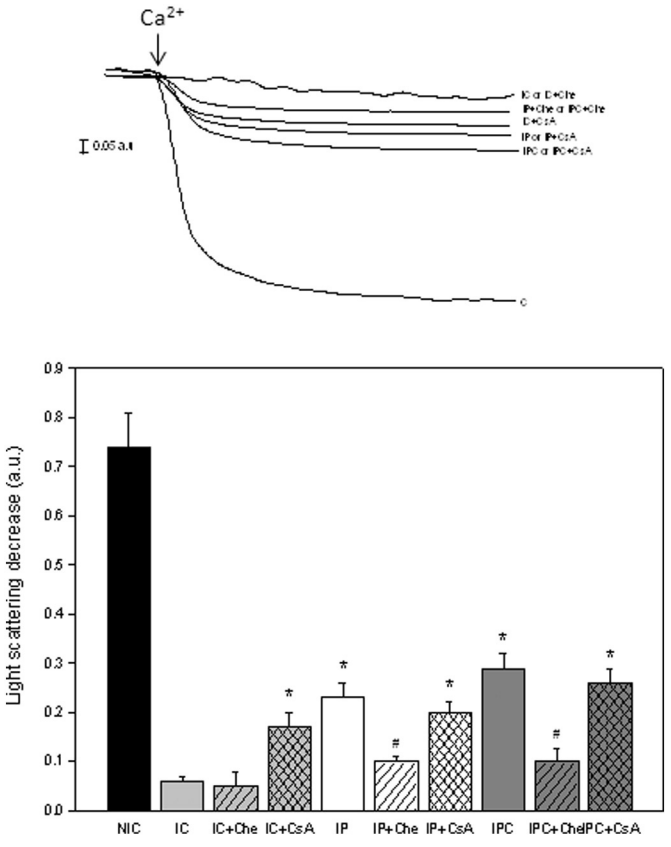

Fig. 8. A: Typical traces produced by 200 μM Ca^{2+} addition to mitochondrial suspensions of the different experimental groups. B: Mean values of the light scattering decreases (LSD) after Ca^{2+} addition, expressed in arbitrary units (a.u.), in non-ischemic control (NIC), ischemic control (IC), preconditioned (IP), postconditioned (IPC) and Che and CsA treated hearts. The response of isolated mitochondria to Ca^{2+} was significantly diminished in IC hearts and was partially recovered after interventions and treatments. Che abolished the favorable changes produced by IP and IPC. *$p < 0.05$ vs. IC; #$p < 0.05$ vs. IP or IPC.

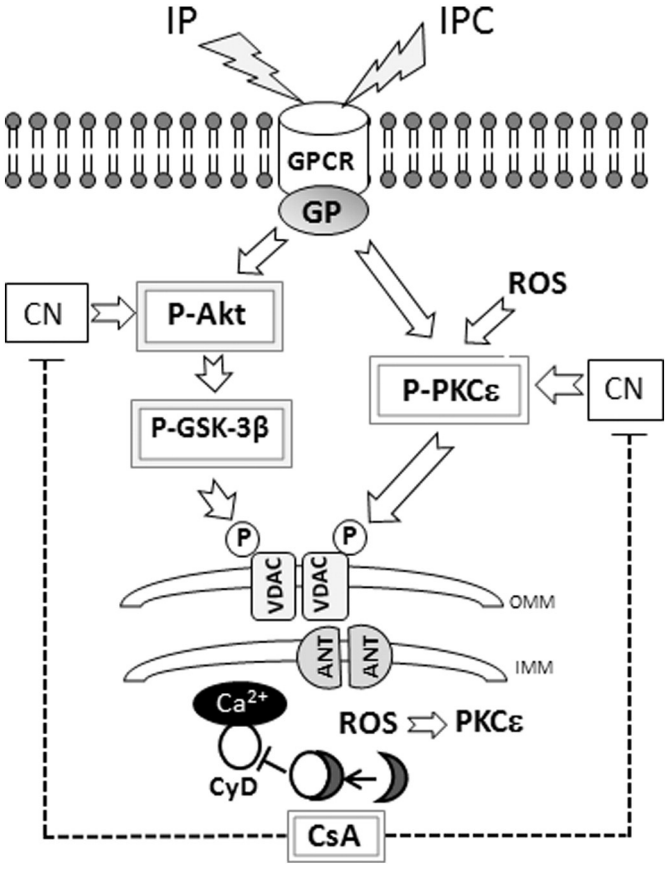

Fig. 9. Proposed scheme for ischemic preconditioning (IP), ischemic postconditioning (IPC) and CsA-mediated cardioprotection. The interaction of agonists released during the brief cycles of IP or IPC protocols with G protein-coupled receptors (GPCR) trigger signaling pathways involving PI3K/Akt/GSK-3β and PKCε activation which have action upon mitochondria. CsA besides sharing IP and IPC cardioprotective cascades has specific actions such as activation of mitochondrial and cytosolic PKCε via ROS signal or by calcineurin (CN) inhibition.

activation could be involved in the reduction of lipoperoxides observed.

It is also accepted that mitochondrial membrane potential (ΔΨm) is disrupted altering over-all energy and redox balance within cardiac myocytes, in response to oxidative stress produced by ischemia–reperfusion. Specifically, under these conditions, ROS build-up can exceed a threshold level that triggers the sequential opening of mitochondrial channels followed by mPTP (Aon et al., 2007) which in turn leads to ΔΨm instability. According to a recent paper (Xie et al., 2014) CsA completely abolished the ΔΨm depolarization induced by H_2O_2 perfusion. Therefore, in our experimental conditions, the normalization of ΔΨm by CsA could be other mechanism contributing to the cardioprotection.

Recognizing to mitochondrial Ca^{2+} overload as a marker for injury, recent results (Dong et al., 2010) suggest that PKCε interacting with Ca^{2+}-sensing receptors protects post-conditioned cardiomyocytes from programmed cell death by inhibiting mitochondria–endoplasmic reticulum crosstalk. This action was also described by CyD inhibition (Paillard et al., 2013; Alam et al., 2015) which would lead to a lesser mitochondrial Ca^{2+} overload, thus improvement of mitochondrial function and diminishing the ROS abrupt production. Moreover, the small amount of ROS could be part of a reverberant loop that reactivates upstream PKC thus contributing to CsA-induced protection. In our experimental conditions these mechanisms were not evaluated but cannot be discarded. Therefore, the inhibition of CyD by CsA would protect against ischemia–reperfusion injury via two synergistic actions (1) the upstream limitation of mitochondrial Ca^{2+} overload by the reduction of Ca^{2+} transfer from endoplasmic reticulum and (2) the downstream prevention of PTP formation via the limitation of CyD binding to the inner mitochondrial membrane.

5. Conclusions

The present study demonstrates that CsA, similarly to IP and IPC exerts PKC-dependent cardioprotective effects in hypertrophied hearts from SHR. Moreover, our data highlight the important role of the physical association P-PKCε/VDAC to preserve the mitochondrial integrity. Our results also suggest that prevention of CyD–ANT interaction, calcineurin inhibition and ROS-mediated PKCε activation are the possible mechanisms involved in the greater limitation of infarct size afforded by CsA (Fig. 9). Finally, the results of this study point to CsA as a promising protective strategy for the ischemic myocardium in hypertensive population.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yexmp.2016.01.009.

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