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Redox signaling in ischemic postconditioning protection involves PKCε and Erk1/2 pathways and converges indirectly in Nrf2 activation

Jorge-Luis Díaz-Ruíz[1], Arturo Macías-López[1], Francisco Alcalá-Vargas, José-Guadalupe Guevara-Chávez, Aída Mejía-Uribe, Alejandro Silva-Palacios, Alejandra Zúñiga-Muñoz, Cecilia Zazueta, Mabel Buelna-Chontal*

Department of Cardiovascular Biomedicine, National Institute of Cardiology, Ignacio Chávez, 14080 Mexico City, Mexico

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ABSTRACT

Ischemic-postconditioning (iPostC) exerts cardioprotection by preserving redox homeostasis in the reperfused heart. This protective effect has been associated with the activation of endogenous antioxidant response driven by transcription factor Nrf2 and with the activation of 'reperfusion injury salvage kinases' (RISK) as PI3K, PKC and Erk1/2. Redox homeostasis is essential for normal cell physiology since reactive oxygen species (ROS) are crucial for processes that involve protein signaling. Thus, it has become clear that not only the perturbation of redox balance to oxidative state is deleterious but also towards a reductive state contributing to pathogenesis of diseases. However, there is still a scarce knowledge about the role of ROS in the cardioprotective signals mediated by RISK in postconditioned hearts. Therefore, we studied the role of ROS as initiator of RISK signaling molecules in iPostC-conferred cardioprotection. With the aim to study the relationship between redox-dependent RISK activation and the downstream activation of the transcription factor Nrf2, we evaluated the effect of redox signaling disruption by the effect of ascorbic acid in iPostC hearts. Our results showed that PKCε and Erk1/2 activation is redox-dependent and that concurs downstream with Nrf2 deficient activation. Besides, using inhibitors we found that neither PI3K nor Erk1/2 are directly related with Nrf2 activation, indicating that these kinases have other targets. We conclude that redox signaling participates in cardioprotection triggered by iPostC through the action of kinase-dependent and -independent mechanisms and concurred with the downstream regulation of Nrf2-mediated antioxidant response to prolonged redox balance during long reperfusion.

1. Introduction

Coronary atherosclerosis is the main cause of coronary artery disease and acute myocardial infarct that is the leading cause of mortality worldwide. Reperfusion of the ischemic tissue can lead to irreversible additional damage [1] mainly due to oxygen re-exposure that stimulates the massive production of reactive oxygen species (ROS) [2–4]. Experimental evidence has demonstrated that the first minutes of reperfusion represents an opportunity window to apply therapies aimed to prevent reperfusion injury [5]. Ischemic postconditioning (iPostC) is a cardioprotective maneuver in which short cycles of ischemia/reperfusion (IR), are applied in the coronary artery before prolonged reperfusion and after a severe ischemic event [6]. This strategy activates an endogenous response in the myocardium that protects against reperfusion injury, which has been tested in several experimental models [7] and in clinical trials [8–11]. Protective mechanisms triggered by iPostC involve the activation of 'Reperfusion Injury Salvage Kinases' (*RISK*) [12,13], that includes the PI3K/AKT (*Phosphatidyl-inositol 3-kinase/protein kinase B*), MEK1/2/Erk1/2 (*Mitogen Activated Kinases/ Extracellular signal Activated Kinases*) [13–15] and the epsilon isoform of protein kinase C (PKCε) pathways [16,17]. Also, iPostC reduces ROS levels, preserves reduced glutathione (GSH) levels [18–20]

Abbreviations: iPostC, Ischemic Postconditioning; asc, Ascorbic Acid; ARE, Antioxidant Response Element; IR, Ischemia and Reperfusion; MEK1/2/Erk1/2, Mitogen activated kinases/ Extracellular signal activated kinases; Nrf2, Transcription nuclear factor E2 related factor 2; PI3K/AKT, Phosphatidyl-isnositol 3-kinase/protein kinase B; PKCε, Protein kinase C, isoform epsilon; RISK, Reperfusion injury salvage kinases; ROS, Reactive oxygen species; U, U0126 (MEK inhibitor); W, Wortmannin (PI3K inhibitor); γ-GCS, gamma-glutamylcysteine synthetase

* Corresponding author at: Department of Cardiovascular Biomedicine, National Institute of Cardiology I. Ch., Juan Badiano #1, Colonia Sección XVI, Tlalpan, 14080, Mexico City, México.
E-mail address: mabel.buelna@comunidad.unam.mx (M. Buelna-Chontal).
[1] These authors contributed equally to this work.

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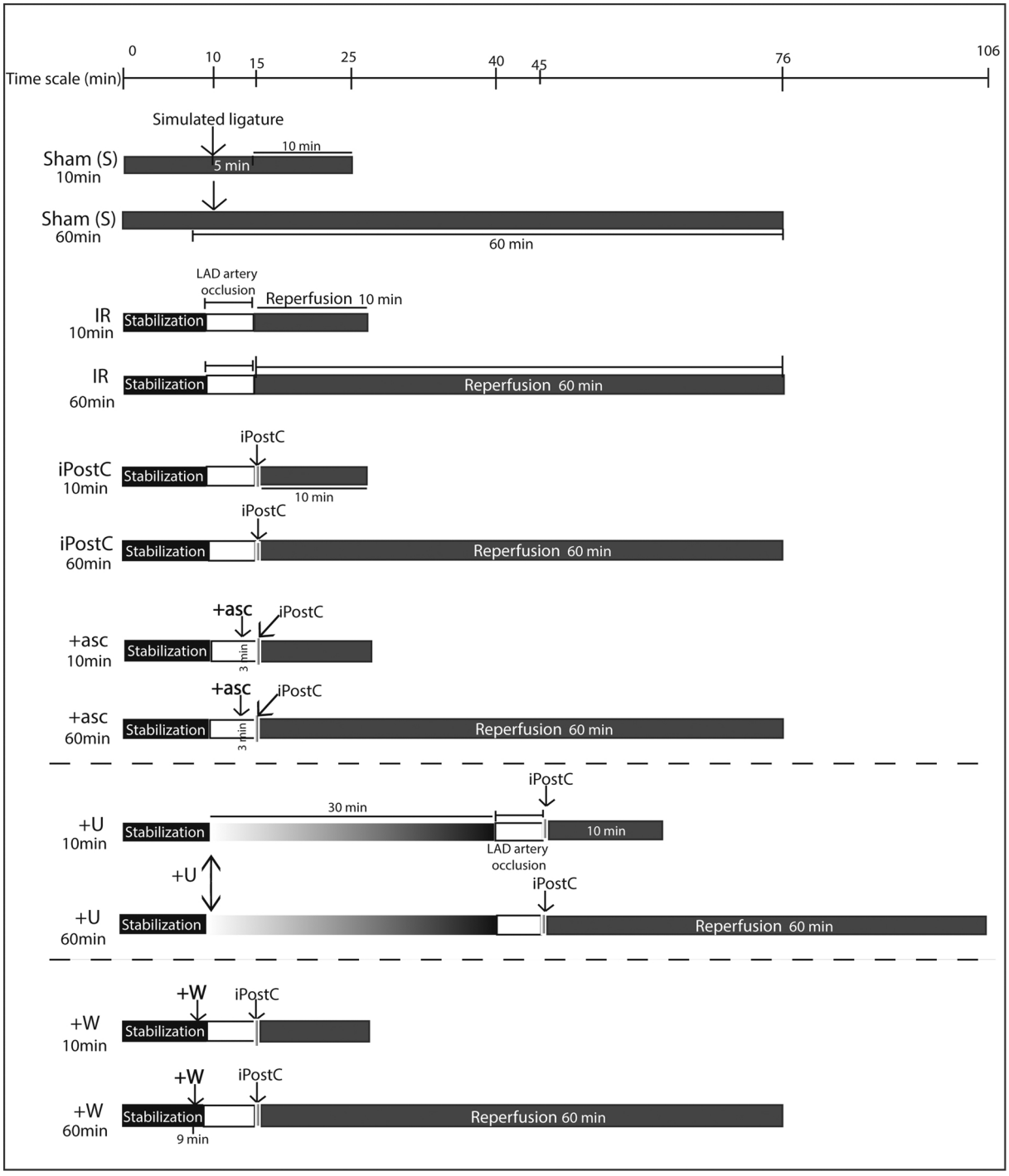

Fig. 1. Experimental protocols.
All experimental groups underwent stabilization for 10 min. The white bars represent coronary artery (CA) occlusion (5 min), followed by 10 min or 60 min of reperfusion (dark bars). iPostC groups received 3 reperfusion/re-occlusion cycles of 10 s each. Where indicated ascorbic acid (+asc), U0126 (+U) and wortmannin (+W) were administered to ischemic postconditioned rats. (2-column fitting figure).

and increases the activity of antioxidant enzymes after activation of transcription nuclear factor E2-related factor 2 (Nrf2) contributing to long-term cardiac protection [21].

Nrf2 contributes to maintain redox homeostasis through regulation of endogenous antioxidant system, which influences to a proper environment for ROS can act as signaling molecules [22,23]. The role of ROS in the protective mechanisms activated by iPostC was firstly reported by Penna et al., [24] who proposed that these molecules are produced by mitochondrial ATP-sensitive K^+ channels (mitoKATP) in a controlled manner and activate PKCε in iPostC hearts. To provide further insight on the role of redox signaling in cardioprotection exerted by iPostC, we applied ascorbic acid (*asc*) to iPostC hearts, in order to study the RISK activation by redox protective signaling and the downstream regulation of transcription factor Nrf2. Our results show that redox signaling exerts regulation on PKCε and Erk1/2 activation and that concurs with the activation of Nrf2, this mechanism appears to be hydrogen peroxide-dependent. We propose that redox signaling participates in cardiac protection by survival kinase-dependent

mechanism that regulates indirectly the downstream Nrf2-mediated antioxidant response which in turn preserves redox homeostasis during long-reperfusion.

2. Material and methods

2.1. Reagents

Chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and Cayman Chemical (Ann Arbor, MI, USA). Polyclonal Antibodies, γ-glutamyl cysteine synthetase (γ-GCS) (ab41463), PHO-PKCε (ab63387), PHO-PI3K (ab182651) and monoclonal antibodies against Nrf2 (ab89443), PI3K p85 (M253, ab86714), PHO-Nrf2 (phospho S40, ab76026) and Lamin-B1 (ab133741), as well as protease and phosphatase inhibitor cocktail (EDTA-free, ab201120), 10× bloking buffer (ab126587) and horseradish peroxidase (HRP)-conjugates secondary antibodies were purchased from Abcam (Cambridge, MA, USA). PHO-Erk1/2 (Thr202/Tyr204, mAb #4370) Erk1/2 (#9102), PHO-MEK1/2 (Ser217/221, mAb #9154) total MEK1/2 (#9122), PKCε (22B10, #2683), hemeoxygenase-1 (HO-1) (#5061) and Erk1/2 inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate also known as U0126 (U) (#9903) were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies raised against, PHO-AKT (C-11, sc-514,032), AKT (B-1, sc-5298) and polyclonal antibody against Nrf2 (H-300, sc-13,032) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotin 3'end DNA labeling kit (89818), chemiluminescent nucleic acid detection module (89880) and lightshift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (20148) were from Thermo Scientific (Rockford, IL, USA). An enhanced chemiluminescence detection system was obtained from Merck Millipore Corp. (Bedford, MA, USA), and the PI3K inhibitor wortmannin (W) (681676) was purchased from Calbiochem (San Diego, CA, USA).

2.2. Methods

Animals experiments were performed in compliance with the ARRIVE guidelines and were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH-US Publications No.8023, revised 1978) and the management of biological residues in accordance to the Norma Oficial Mexicana NOM-062-200-1999. Experimental protocol design was mostly according to the "*Practical guidelines for rigor and reproducibility in preclinical and clinical studies on cardioprotection*" [25]. Male Wistar rats (300 g) were used in this study. The animals were injected with 63 mg/Kg *i.p.* sodium pentobarbital and 1.7 U/Kg *i.p.* heparin and assessed for complete lack of pain response by determining pedal withdrawal reflex. Each animal was intubated through an inserted cannula into the trachea and connected to a respirator for small animals (Harvard Apparatus, Holliston, MASS, USA). Lateral thoracotomy was performed to expose the heart and to occlude 5 min the main branch of the left coronary artery, near its origin beneath the left atrial appendage with a 6–0 polypropylene suture slip knot, which was timely released to reperfusion of ischemic tissue [26,27]. The 5 min occlusion of the main coronary artery has been shown to produce severe cardiac injury [21,28] and we compared the infarct size obtained with this protocol against 30 min occlusion of left anterior descending artery (Suppl. Fig. 2A and B). Hemodynamic parameters were measured using a pressure-volume catheter (Mikro-Tip; Millar) inserted into the left ventricle cavity and subsequent analysis was performed with PVAN Ultra software (Millar, Houston, Tx, USA).

2.3. Surgical procedures

The experimental protocols are described in Fig. 1. All groups were stabilized during 10 min, a simulated coronary artery occlusion was performed, by placing the polypropylene suture around the artery without occlusion in the sham groups during short term, 10 min after simulated occlusion ($n = 5$) or long term, 60 min after simulated occlusion ($n = 5$). The IR groups were subjected to 5 min occlusion of the main branch of the left coronary artery producing immediate pallor of the left ventricle wall. Blood reflow was restored during 10 ($n = 5$) or 60 min ($n = 10$) and hemodynamic data were recorded. In the iPostC groups blood flow was restored during 10 ($n = 5$) or 60 min ($n = 10$) after 3 cycles of 10 s of reperfusion/10 s of re-occlusion [29–31]. The antioxidant ***asc*** 100 mg/Kg ($n = 10$) [32] and the inhibitors **U** 1.27 mg/Kg ($n = 10$) [33] and **W** 50 nM (100 μL; $n = 10$) [34], were injected directly into the muscle wall of the left ventricle of iPostC hearts. The antioxidant ***asc*** was administered at min 3 of coronary occlusion, whereas **U** group was stabilized and then was injected followed by 30 min before coronary occlusion and **W** hearts were applied at min 9 of stabilization.

2.4. Infarct size measurement

At the end of the protocol, the coronary artery was re-occluded for injection into left ventricle of 1 mL 5% Evan's blue to determine the extent of risk area [35]. Hearts from each experimental group were excised ($n = 25$) and frozen at −20 °C for 20 min, then were cut into 2-mm transverse slices for incubation in 1% 2,3,5-triphenyl tetrazolium chloride in sodium phosphate buffer (pH 7.4) during 20 min at 37 °C in constant agitation. Finally, the slices were immersed in a preservative solution of phosphate buffer saline containing 0.01% sodium azide, to enhance the contrast between non-risk zone (blue), risk zone (pale and red stain) and infarcted area (white) [21]. All slices were photographed and using the software Image J (NIH, Bethesda, MD, USA) was calculated the percentage of the infarct size (IS) normalized per area at risk (AAR) of left ventricle.

2.5. Hydrogen peroxide quantification

Immediately, after in vivo protocol left ventricles were obtained from each experimental condition reperfused for 10 min ($n = 20$), followed by homogenization of fresh cardiac tissue using Dulbecco's phosphate-buffered saline (DPBS) (2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4, 136.9 mmol/L NaCl, 8.9 mmol/L $Na_2HPO_4 \cdot 7H_2O$ and 1% nonidet P-40 and protease inhibitor cocktail), then centrifuged at 4000*g* at 4 °C during 15 min and the supernatant was collected. The assay was based on the amplex red reaction with hydrogen peroxide in the presence of HRP [36]. Cardiac homogenates were mixed with 10 μmol/L of 10-acetyl-3,7-dihydrophenoxazine and 0.2 U/mL HRP in Krebs-Ringer's phosphate glucose buffer (145 mmol/L NaCl, 5.7 mmol/L NaH_2PO_4, 4.86 mmol/L KCl, 0.54 mmol/L $CaCl_2$, 1.22 mmol/L $MgSO_4$, 5.5 mM glucose) and incubated in darkness at 37 °C for 60 min. Fluorescence was detected at 590 nm (emission) using an excitation wavelength of 530 nm in an Spectro-fluorophotometer (Shimadzu RF5000U). Data were obtained from a hydrogen peroxide standard curve and results were expressed as nmol hydrogen peroxide per mg of total protein.

2.6. Malondialdehyde determination assay

Samples obtained previously from left ventricles homogenates reperfused 10 min ($n = 20$), were incubated in a reaction mixture containing 15.4 mM 1-methyl-2-phenylindole in the presence of chlorhydric acid, at 45 °C during 40 min [37]. Then centrifuged at 3000*g* for 5 min and the optical density of the supernatant was finally measured spectrophotometrically (Biotek Synergy HTX multi-mode reader) at 586 nm. A standard curve of tetrametoxypropane was used and data were expressed as nmol malondialdehyde (MDA) per mg of total protein.

Table 1
Hemodynamic data obtained at the beginning of the stabilization period (baseline) and after 60 min of reperfusion.

	Baseline						Reperfusion					
	LVP (mmHg)	LVDP (mmHg)	HR (beats/min)	EDP (mmHg)	max dP/dt (mmHg/s)	min dP/dt (mmHg/s)	LVP (mmHg)	LVDP (mmHg)	HR (beats/min)	EDP (mmHg)	max dP/dt (mmHg/s)	min dP/dt (mmHg/s)
S	106 ± 19	95 ± 14	328 ± 70	4.2 ± 1.5	6684 ± 1968	−4253 ± 1639	127 ± 20	117 ± 21	272 ± 49	5.7 ± 2.2	5271 ± 1673	−3849 ± 1354
IR	107 ± 11	101 ± 9	338 ± 26	4.3 ± 1.2	7598 ± 674	−4787 ± 746	57 ± 22[&]	59 ± 28[&]	272 ± 43	15.5 ± 4.6[&]	1158 ± 705[&]	−404 ± 178[&]
iPostC	102 ± 16	98 ± 11	326 ± 32	4.6 ± 1.3	7773 ± 1247	−4687 ± 1526	120 ± 14[#]	113 ± 11[#]	329 ± 17	5.1 ± 1.8[#]	6180 ± 1026[#]	−4412 ± 1605[#]
+ asc	111 ± 12	106 ± 14	355 ± 25	4.8 ± 1.3	7267 ± 671	−4087 ± 872	57 ± 21[@]	38 ± 17[@]	210 ± 39[@]	12.3 ± 5.3[@]	1319 ± 622[@]	−925 ± 492[@]

Left ventricular pressure (LVP), left ventricular developed pressure (LVDP), heart rate (HR), left ventricular end diastolic pressure (EDP), contractility index (max dP/dt) and relaxation index (min dP/dt). Values are the mean of at least 5 experiments ± S.D. Sham (S), ischemic-reperfused (IR), ischemic post-conditioned (iPostC), iPostC hearts plus ascorbic acid (+asc).
[&] $p < .01$ vs. Sham.
[#] $p < .01$ vs. IR.
[@] $p < .01$ vs. iPostC.

2.7. Reduced glutathione content

Monochlorobimane (1 mM) was used to form a stable fluorescent adduct with glutathione (GSH) in the presence of glutathione S-transferase (1 U/mL). The assay was performed as described by Fernández-Checa & Kaplowitz [38]. Samples obtained previously from left ventricles homogenates reperfused 10 min ($n = 20$), were incubated in the reaction mixture at 37 °C during 30 min and the fluorescence was assessed at an excitation wavelength of 385 nm and emission wavelength of 478 nm (Biotek Synergy HTX multi-mode reader). Results were calculated according to standard curve for GSH (nmol) and then normalized per mg of protein.

2.8. Measurement of PKC activity

A commercial kit was used to measure PKC activity in samples of left ventricles homogenates obtained previously ($n = 20$) (PepTag assay, Promega, Madison, WI, USA). Briefly, the change in net charge of a specific peptide substrate for PKC shifts its mobility in agarose gel, allowing to distinguish between the phosphorylated and the non-phosphorylated peptide. Each band was excised from the gel and melted to quantify the content of the peptide by spectrophotometry at 570 nm. One unit of PKC activity is defined as the number of nanomoles of phosphate transferred to substrate per minute per milliliter.

2.9. Nuclear protein extraction

Frozen samples from left ventricles reperfused for 60 min ($n = 30$) were used for nuclear protein extraction as described by Kuster et al., [39]. Left ventricle tissues were pulverized, and nuclear protein extraction was obtained at 4 °C using the NE-PER kit instructions (Thermo Scientific, Rockford, IL, USA).

2.10. Immunoprecipitation assay

Cardiac homogenates from left ventricles reperfused 10 min obtained formerly (1.5 mg; $n = 20$) were incubated with 4 μg of primary antibody to Nrf2 (H-300; Santa Cruz Biotechnology, Santa Cruz, CA.) during 3 h, at 4 °C in a rotary mixer. Then, were added 50 μL of protein G-sepharose beads (slurry) and incubated overnight at 4 °C in a rotary mixer. Afterward the samples were centrifuged at 1200*g*, for 30 s at 4 °C. The beads were washed twice with 50 mM Tris-HCl, 120 mM NaCl, 0.5% Igepal, 100 mM NaF pH 8.0 and finally centrifuged at 1200*g* at 4 °C for 30 s. The beads were collected and 5× SDS sample buffer was added to perform western-blot assays.

2.11. Western blotting

Left ventricles homogenates formerly obtained (reperfused 10 min; $n = 20$) or nuclear protein samples ($n = 30$) were used for western blot analysis. Proteins were separated alternatively in 8%, 10% or 15% SDS-PAGE gels and electro-transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat milk or blocking buffer 1 X (Abcam, Cambridge, MA, USA) for 1 h and then incubated with primary antibodies at 4 °C overnight. After that blots were incubated with secondary antibodies coupled to HRP and detected by chemiluminescence.

2.12. Electrophoretic mobility shift assay (EMSA)

The oligonucleotide containing the sequence of the 'antioxidant response element' (ARE) (GATCTTTTA*TGCTGAGTCA*TGGTTT) was manufactured by Sigma Aldrich (Toluca, Mexico) and was further biotin-labeled with a commercial kit (Thermo Scientific, Rockford, IL, USA). Binding reactions were carried out at room temperature for 60 min in the presence of 50 ng/μL poly (dI·dC), 0.05% nonidet P-40,

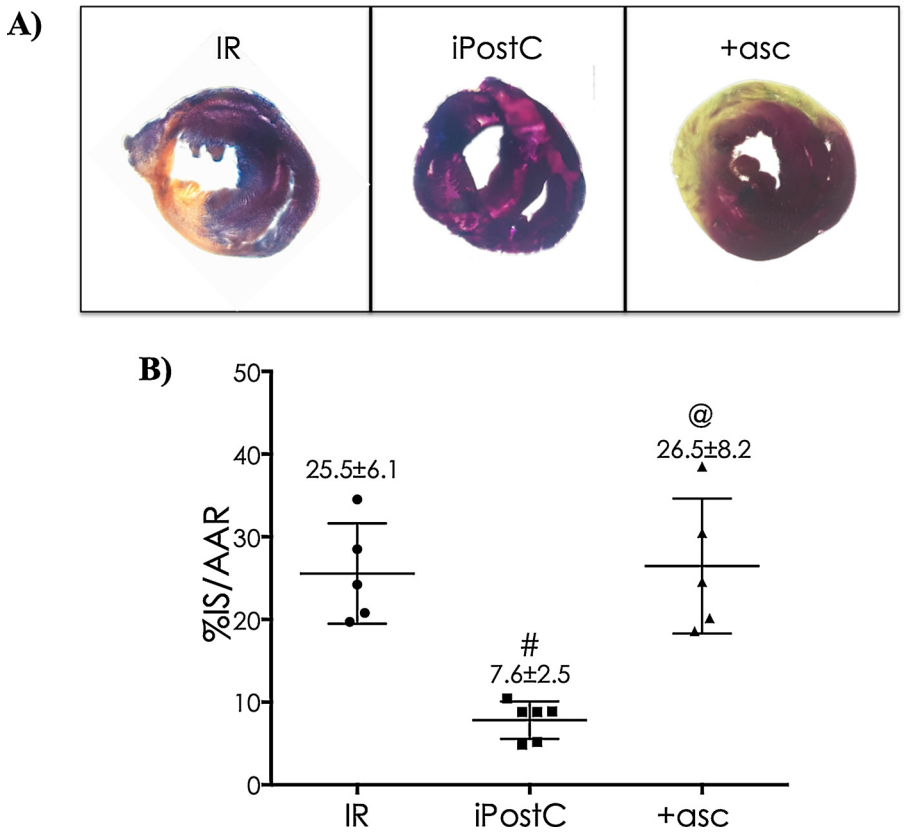

Fig. 2. Infarct size measurement.
A) Representative images of heart slices corresponding to IR, iPostC and + asc groups. Blue stain, non-risk area; Red stain, viable tissue; Pale stain, infarcted area. B) Infarct size was calculated as percentage related to area at risk. IS = Infarct size; AAR = area at risk; Data represents the mean of 5 independent experiments ± S.D. #$p < .01$ vs. IR; @$p < .01$ vs. iPostC. (1-column fitting column), color figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5% glycerol, 10 mM EDTA, 5 mM $MgCl_2$, 0.05 M KCl in 1× binding buffer using 20 fmol of biotin-end-labeled-ARE (B-ARE) and 10 μg of nuclear extract.

The competition assay was performed by the addition of an excess of unlabeled-ARE that was incubated for 30 min before the addition of the B-ARE sequence. The reaction mixtures were loaded onto native 5% polyacrylamide gel pre-electrophoresed for 60 min in 0.5× Tris-Borate-EDTA (TBE) buffer, electrophoresed at 100 V and finally transferred onto a positively charged nylon membrane in 0.5× TBE at 380 mA for 30 min. Transferred DNA was cross-linked to the membrane and chemiluminescent detection was achieved using HRP-conjugated streptavidin.

2.13. Statistical analysis

Data are expressed as the mean ± S.D. Differences in all variables were assessed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison's test. A statistically difference of $p < .05$ or $p < .01$ was considered significant. We used the software GraphPad Prism version 6 for data analysis.

3. Results

3.1. The iPostC exerted protection by maintaining cardiac function and prevented infarct size, these effects were abolished by the presence of ascorbic acid

Left ventricular pressure (LVP), left ventricular developed pressure (LVDP), heart rate (HR), left ventricular end diastolic pressure (EDP), contractility (max dP/dt) and relaxation (min dP/dt) indexes were measured during open chest surgery. Baseline values and those registered after 60 min of reperfusion are shown in Table 1. No significant differences were obtained in the baseline registers of the different experimental groups. At the end of reperfusion LVP diminished in the IR group as compared to the sham group (57 ± 22 mmHg vs. 127 ± 20 mmHg; $p < .01$). The iPostC maintained LVP at similar values to those registered in the sham group (120 ± 14 mmHg; 127 ± 20 mmHg) and significantly higher than those of the IR group (127 ± 14 mmHg vs. 57 ± 22 mmHg; $p < .01$). The *asc* addition in the iPostC hearts resulted in a significant depletion of LVP (57 ± 21 mmHg). LVDP followed a similar pattern in the IR group was significantly lower than in the sham group (59 ± 28 mmHg vs. 117 ± 21 mmHg; $p < .01$), was increased in the iPostC group (113 ± 11 mmHg) and the *asc* addition decreased LVDP values (38 ± 17 mmHg).

HR values were similar between the sham, IR and iPostC groups. A 36.2% decrease was observed in the *asc group* in comparison with the iPostC group (210 ± 39 beats/min vs. 329 ± 17 beats/min; $p < .01$). On the other hand, EDP was significantly higher in the IR in comparison to sham (15.5 ± 4.6 vs. 5.7 ± 2.2 mmHg; $p < .01$), was maintained in iPostC group (5.1 ± 1.8 mmHg) and was increased in the +asc group (12.3 ± 5.3 mmHg). The left ventricle systolic function or max dP/dt was recovered efficiently in the iPostC hearts in contrast to the IR group (6180 ± 1026 mmHg/s vs. 1158 ± 705 mmHg/s; $p < .01$) and was affected by *asc* addition (+asc) (1319 ± 622 mmHg/s vs. 6180 ± 1026 mmHg/s; $p < .01$). The values corresponding to min dP/dt was improved in iPostC hearts as compared with IR hearts (−4412 ± 1605 mmHg/s vs. −404 ± 178 mmHg/s; $p < .01$). The *asc* addition rendered higher relaxation values than iPostC (−925 ± 492 mmHg/s vs. −4412 ± 1605 mmHg/s; $p < .01$). Due to the *asc* addition in iPostC hearts the cardiac function was hindered, probably by perturbation of redox homeostasis disturbing in consequence activation of protective mechanisms. Conversely the effect of *asc* in IR hearts had indeed protective effects by alleviating oxidative stress in such condition (Suppl. Table 1). Besides the protective effect of iPostC was able to limit IS caused by reperfusion injury by a clear diminishment of infarct area relative to AAR in hearts with iPostC in comparison to IR, this

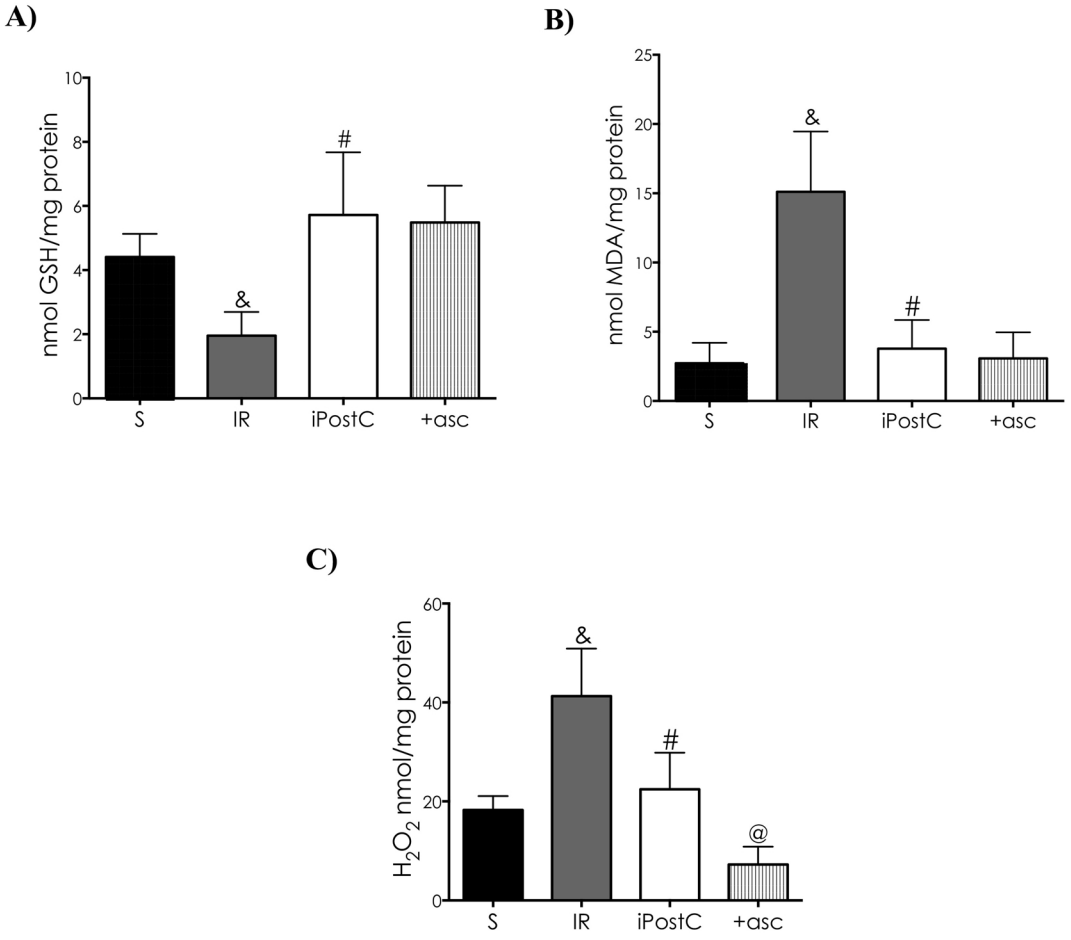

Fig. 3. Ischemic postconditioning preserves redox homeostasis.
(A) Reduced glutathione (GSH) content expressed as nmol per mg protein. (B) Malondialdehyde (MDA) content expressed as nmol/mg protein. (C) Quantification of hydrogen peroxide (H_2O_2) expressed as nmol per mg protein. Data are the mean of at least 5 experiments. $^{\&}p < .01$ vs. Sham; $^{\#}p < .01$ vs. IR and $^{@}p < .01$ vs. iPostC. (1.5 column fitting image).

protective effect was also abolished by the *asc* addition in iPostC hearts (Fig. 2). These results indicate that redox homeostasis is crucial for a proper cardiac function afforded by iPostC application.

3.2. Application of iPostC preserved redox homeostasis and was disturbed by addition of ascorbic acid

GSH is a reliable indicator of the intracellular redox balance. GSH content decreased in IR hearts after 10 min of reperfusion as compared with the sham group (1.96 ± 0.7 vs. 4.4 ± 0.7 nmol/mg protein; $p < .01$), whereas in hearts subjected to iPostC, GSH content increased to 5.7 ± 1.9 nmol/mg. In the iPostC hearts with *asc* addition (+asc) the GSH level was not altered and remained at a similar level to iPostC (5.5 ± 1.1 nmol/mg) (Fig. 3A). Oxidative stress damage was evaluated by MDA assessment, a toxic byproduct of lipid peroxidation. The loss of redox balance by IR was confirmed since MDA levels was increased as compared with sham group (15.1 ± 4.3 vs. 2.7 ± 1.5 nmol/mg protein; $p < .01$), whereas the levels of MDA in iPostC hearts were lower than those observed in the IR group (3.8 ± 2.1 nmol/mg protein; $p < .01$). MDA levels in the +asc group did not change in comparison to those levels of iPostC group (3.1 ± 1.9 nmol/mg protein; Fig. 3B). Additionally, we quantified hydrogen peroxide after early reperfusion (10 min). Hydrogen peroxide concentration increased 2.3-fold in the IR group in comparison to the sham group as represented in Fig. 3C (41.3 ± 9.6 vs. 18.3 ± 2.8 nmol/mg protein; $p < .01$); this increase was prevented by iPostC that was 1.8-fold lower than in IR hearts (22.5 ± 7.4 vs. 41.3 ± 9.6 nmol/mg protein; $p < .01$). These data indicate that IR disrupts the redox balance towards pro-oxidative state during early reperfusion, conversely to iPostC, which preserved a redox balance, since GSH levels were higher, lipid peroxidation was prevented, and hydrogen peroxide was decreased in comparison to IR group. The chemical nature of hydrogen peroxide determines its properties as a triggering molecule in redox signaling. We observed that ***asc*** addition to iPostC hearts (+asc) dramatically decreased hydrogen peroxide concentration by 3.1-fold in comparison to iPostC group (7.2 ± 3.6 vs. 22.5 ± 7.4 nmol/mg protein; $p < .01$) and this concurred with the impairment of cardiac function previously shown in Table 1, indicating that hydrogen peroxide is involved in cardiac protection afforded by application of iPostC.

3.3. In iPostC hearts the activation of PKC and Erk1/2 was redox-dependent but not PI3K/AKT

To investigate the role of redox signaling on RISK activation, we assessed PKC activity in the +asc group. Increased PKC activity in iPostC hearts as compared with the IR group was abolished by the *asc* addition (Suppl. Fig. 1A and B). To determine if the diminishment observed in PKC activity corresponding to +asc group correlated with changes in PKCε activation, we evaluated its phosphorylation (PHO-PKCε) by western blotting. As shown in Fig. 4A, PHO-PKCε decreased in IR hearts in comparison with the sham group ($p < .01$). In contrast, the application of iPostC increased its phosphorylation as compared to IR hearts ($p < .01$), whereas *asc* addition hindered the activation of

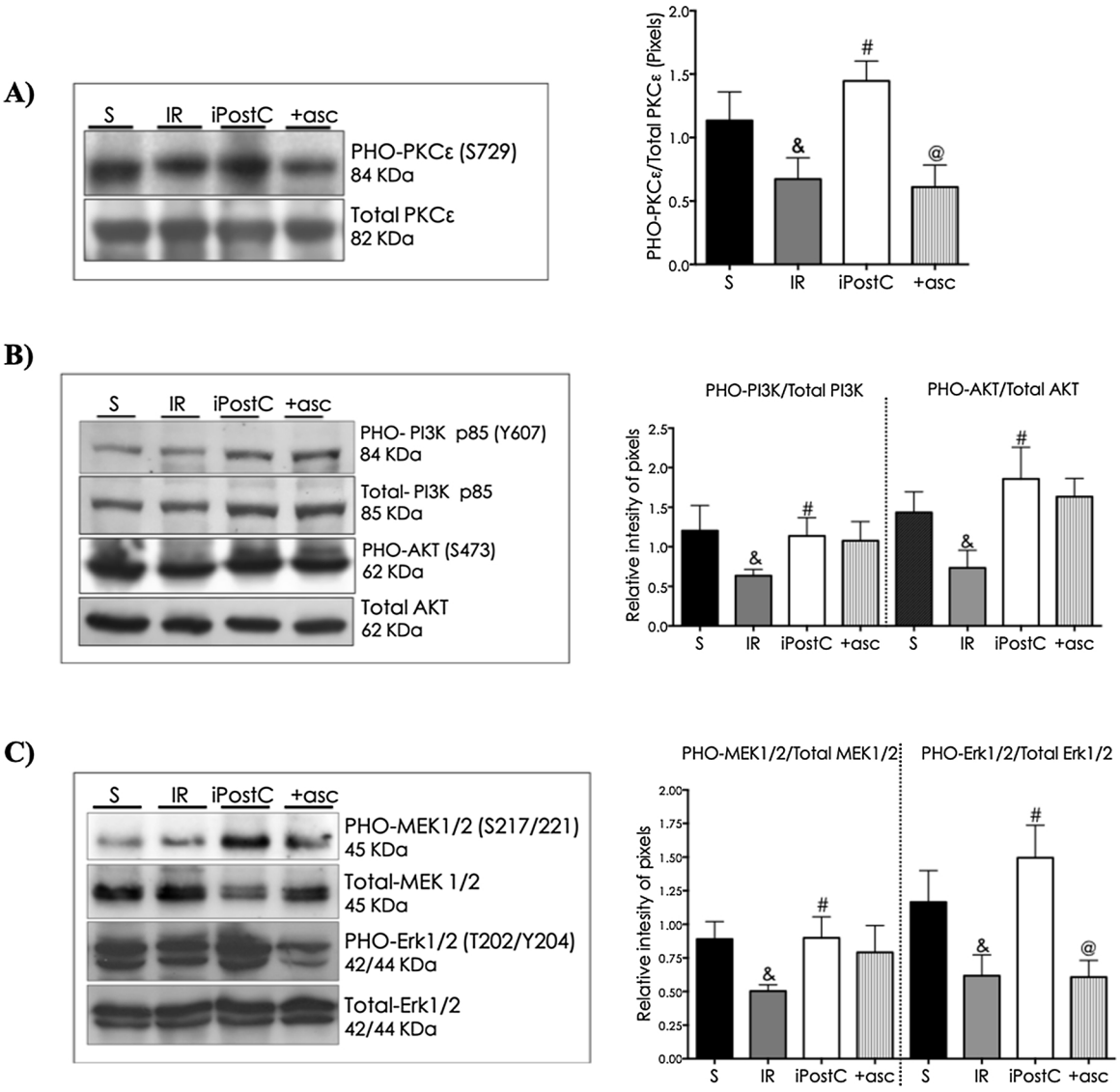

Fig. 4. PKCε and Erk1/2 activation in iPostC hearts was abolished by ascorbic acid.
(A) PHO-PKCε and PKCε content were immunodetected. The content corresponding to phosphorylated PKC was normalized to total PKCε. (B) Representative blots of total and phosphorylated PI3K and AKT (C) PHO-Erk1/2 and PHO-MEK normalized to their corresponding total protein. Data represents the mean of at least 5 experiments ± S.D. $^{\&}p < .01$ vs. Sham; $^{\#}p < .01$ vs. IR and $^{@}p < .01$ vs. iPostC. (1.5-column image).

PKCε observed in iPostC hearts ($p < .01$). We also measured the phosphorylation of PI3K/AKT and MEK1/2-ERK1/2 in iPostC hearts in such conditions (Fig. 4B, C). Both PHO-PI3K and PHO-AKT levels decreased in the IR group as compared to the sham group ($p < .01$) and were recovered by iPostC ($p < .01$). On the contrary to that observed with PKCε, no changes between iPostC and + asc groups were detected neither AKT nor PI3K phosphorylation. We also observed that PHO-MEK1/2 ($p < .01$) and PHO-Erk1/2 ($p < .01$) decreased in IR group in comparison to sham group and both kinases augmented in the iPostC group ($p < .01$). Although no changes in MEK activation were induced by *asc* addition in iPostC hearts, indeed decreased Erk1/2 activation as compared to iPostC hearts ($p < .01$). Our data suggests that PI3K, AKT and MEK activity do not depend of redox signaling, in contraposition with that observed for Erk1/2 activity, which is redox-dependent and such activation is independent of the canonical pathway mediated by MEK. Overall, these results imply that the cardiac dysfunction induced by *asc* in iPostC hearts likely due to a partial inhibition of the protective redox-signals involving PKCε and Erk1/2 and suggests that hydrogen peroxide could be a trigger of such condition.

3.4. Inhibition of redox signaling in iPostC hearts abolished Nrf2 activation

The nuclear accumulation of transcription factor Nrf2 after 60 min of reperfusion is shown in Fig. 5A. Nuclear content of Nrf2 was lower in the IR group than in the sham group ($p < .01$), whereas iPostC increases the content of nuclear Nrf2 in comparison to IR ($p < .01$) and this effect was diminished in presence of *asc* (+asc) ($p < .05$).

EMSA assay was performed using nuclear extracts of hearts after 60 min of reperfusion to evaluate Nrf2 binding to B-ARE sequence in the different experimental groups. In Fig. 5B is shown that iPostC increased the formation of the complex Nrf2:B-ARE in comparison with IR, (upper shift band in lane 4 and 8 vs. 3 and 7), this effect was avoided by *asc* addition in the iPostC hearts (lanes 5 and 9 vs. 4 and 8). These results indicate that redox signaling is involved in the downstream Nrf2 nuclear accumulation and further ARE-binding.

3.5. Role of MEK/Erk1/2 signaling in Nrf2 activation by iPostC

To determine the correlation between MEK1/2-Erk1/2 phosphorylation and downstream Nrf2 activation we used the specific MEK1/2

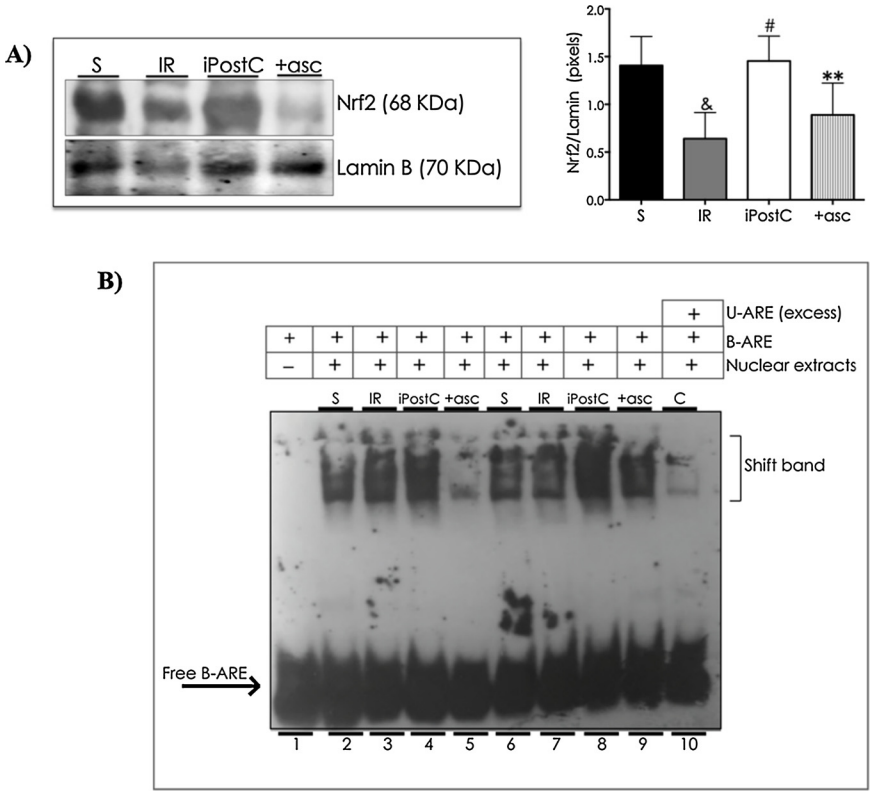

Fig. 5. In iPostC hearts ascorbic acid abolished Nrf2 nuclear translocation and its transcriptional activity.
(A) Nuclear content of Nrf2 related to Lamin B. Data are the mean ± S.D. of 5 experiments. $^{\&}p < .01$ vs. Sham; $^{\#}p < .01$ vs. IR and $^{**}p < .05$ vs. iPostC. (B) Transcriptional activity was assessed by electrophoretic mobility shift assay (EMSA) in all experimental groups (after 60 min reperfusion). Nrf2 binding to B-ARE (ARE-biotin labeled) forms a shift band at the top of the gel. In the lane 1 it is shown the migration of free B-ARE in the non-denaturing gel of polyacrylamide. Lanes 2–9 show the Nrf2:B-ARE complex in sham (S), ischemic-reperfused (IR), ischemic post-conditioned (iPostC) and post-conditioned hearts treated with ascorbic acid (+asc). Lane 10 shows the competition assay in which an excess of unlabeled ARE (U-ARE) was included. The image is representative of 6 independent experiments. (1.5-column fitting figure).

inhibitor, U. The pharmacological inhibition of Erk1/2 signaling in the iPostC hearts, affected the heart performance during reperfusion, LVP, LVDP, HR, EDP, contractility and relaxation indexes were altered in comparison to iPostC hearts (suppl. Table 1). In this sense, there was also an increase of 4.1-fold of IS compared to iPostC group (Fig. 8A, B). In Fig. 6A is shown PHO-MEK and PHO-Erk1/2 content in iPostC hearts treated with U after 10 min of reperfusion. A significant decrease in the phosphorylation of both kinases was observed in the IR group as compared with sham hearts ($p < .01$), application of iPostC increased the levels of PHO-MEK1/2 and PHO-Erk1/2 in comparison to IR group ($p < .01$), whereas both kinases were inhibited by U administration (+U; $p < .01$). We observed that MEK/Erk1/2 inhibition in the +U group did not decrease Nrf2 phosphorylation at Ser40 at early reperfusion (Fig. 6B). To determine if Erk1/2 inhibition could exert a late effect on Nrf2 activation, we also evaluated Nrf2 nuclear accumulation and binding to B-ARE sequence after 60 min of reperfusion in iPostC hearts. Erk1/2 inhibition in iPostC hearts (+U) did not limit Nrf2 nuclear accumulation in comparison to iPostC group (Fig. 6C). Fig. 6D shows a representative image of EMSA assay. It is observed that the basal binding of Nrf2 to the ARE sequence (lanes 2 and 5) decreased in the IR group (lanes 3 and 6), increased in the iPostC group (lanes 4 and 7) and was maintained in presence of Erk1/2 inhibitor (+U) (lanes 8 and 9).

3.6. Role of PI3K/AKT signaling in Nrf2 activation by iPostC

We also evaluated the possible correlation between PI3K/AKT and downstream Nrf2 activation using W, a specific inhibitor of this pathway. The pharmacological inhibition of PI3K/AKT signaling in the iPostC hearts (+W), altered the following hemodynamic parameters during reperfusion, LVP, LVDP, HR, EDP, contractility and relaxation indexes in comparison to iPostC hearts (suppl. Table 1). Besides the PI3K/AKT inhibition (+W) increased 2.9-fold the infarcted area in comparison to iPostC hearts ($p < .05$) (Fig. 8A and B). PI3K and AKT phosphorylation are shown in Fig. 7A; after 10 min of reperfusion the activation of PI3K and AKT of IR group decreased in comparison to sham ($p < .01$), whereas both kinases augmented their phosphorylation in iPostC hearts in comparison to IR ($p < .01$) and PI3K/AKT inhibition in the iPostC hearts (+W) blocked such increase ($p < .01$). On the other hand, although Nrf2 phosphorylation concurred with the decrease of PI3K/AKT activity by W in iPostC hearts at early reperfusion (Fig. 7B), the nuclear accumulation of Nrf2 was not decreased and the binding to ARE sequence was even higher than iPostC group (Fig. 7C and D). To corroborate these results, we measured the content of two antioxidant enzymes transcriptionally regulated by Nrf2. In Fig. 7E is shown content corresponding to γ-GCS and HO-1, both were increased in iPostC hearts in comparison with the IR group ($p < .01$), however the effect of PI3K inhibition in iPostC hearts did not modify the antioxidant protein levels.

4. Discussion

Redox imbalance leading to oxidative injury is related with myocardial cell death and impairment of heart function during reperfusion, whereas strategies that preserves redox homeostasis allows activation of survival signaling pathways to maintain a proper heart function. Our results showed the application of iPostC is crucial to maintenance of redox balance as a part of cardiac protection owing to GSH levels were preserved and MDA was not increased after the first minutes of reperfusion. In the iPostC hearts with *asc*, neither GSH nor MDA levels were significative changed, indicating that the effects we found on signaling was solely due to *asc* presence in the cell rather than promoting a reductive state through GSH increase. It has been well established that ROS can act as second messengers. Hydrogen peroxide is the main molecule in redox signaling, as it is capable to oxidize a wide range of substrates by reacting with specific thiols, that act as sensors to transmit the redox signal [40]. Now can recognize also that ROS can act as second messengers in heart protection [41–43] and addition of exogenous antioxidants remarkably has abrogated the cardioprotective effects of strategies such as iPostC, likely by promoting a transient reductive environment [24,44]. Noteworthy, our data shows the antioxidant *asc* depleted hydrogen peroxide levels in the iPostC hearts and

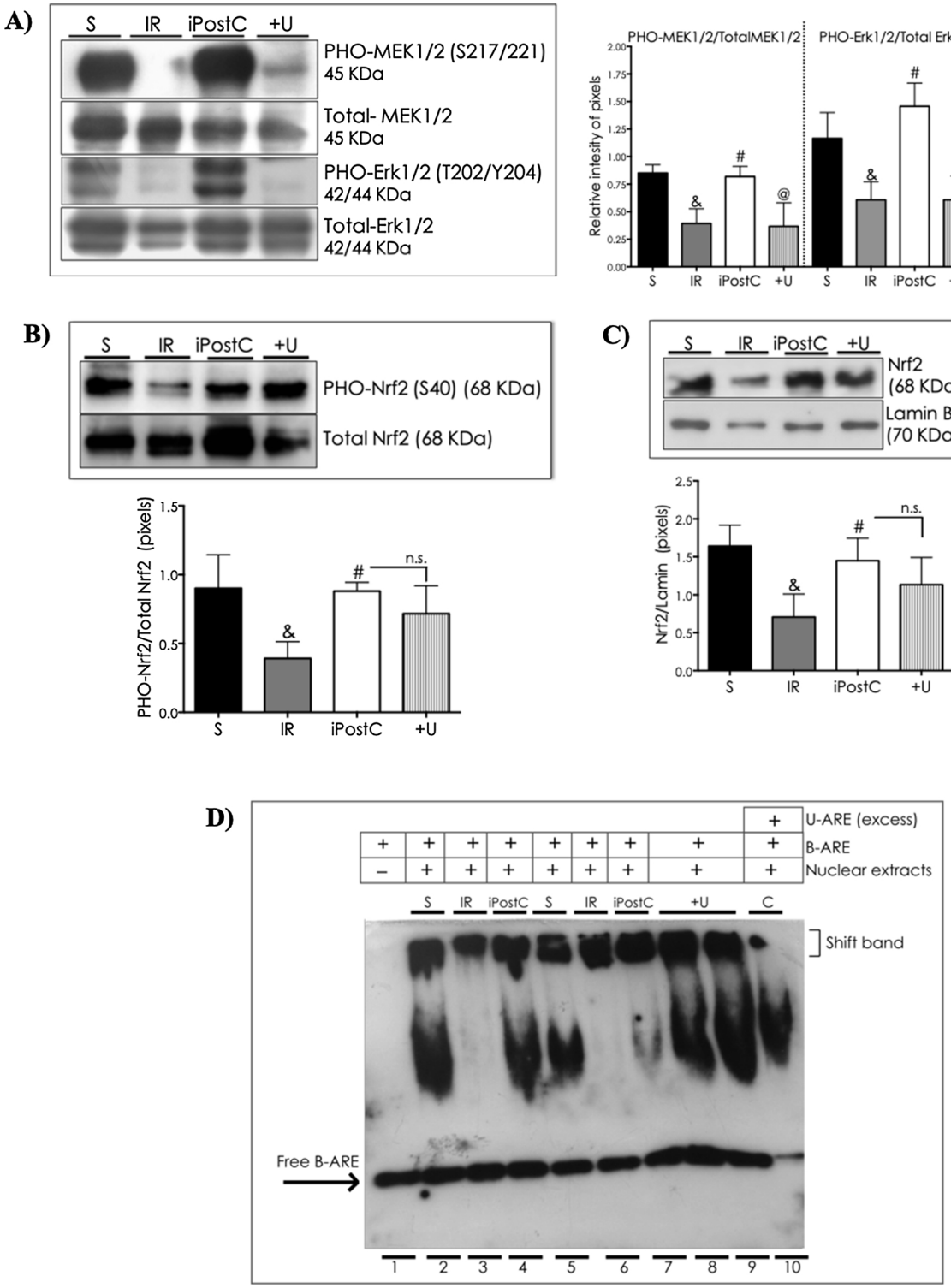

Fig. 6. Nrf2 transcriptional activation is independent of Erk1/2 signaling in iPostC hearts.
(A) PHO-MEK and PHO-Erk1/2 in the presence of U0126 (+U). (B) PHO-Nrf2 normalized to total-Nrf2 in reperfused groups for 10 min. (C) Nrf2 content normalized with Lamin B in nuclear fractions. Graphics show the mean data of at least 5 experiments ± S.D. $^{\&}p < .01$ vs. Sham; $^{\#}p < .01$ vs. IR; $^{@}p < .01$ vs. iPostC and non-significant: n.s. (D) Representative EMSA of 6 different experiments, showing Nrf2:B-ARE complex formation. Lane 1 corresponds to free B-ARE migration. Lanes 2–9 show complex formation of sham (S), ischemic-reperfused (IR), ischemic post-conditioned (iPostC) and post-conditioned hearts treated with inhibitor U0126 (+U). Lane 10 shows competition assay (*C*). (1.5-column fitting figure).

this concurred with the impairment of heart function. Indeed, disruption of redox homeostasis towards a pro-reductive state has been described as 'reductive stress' [45], abolishing the redox signaling causing detrimental and pathological effects in hearts [46–48]. As prolonged and overwhelming unbalance either towards oxidative or reductive states are harmful for the cell, it can be speculated that the disappointing results exerted by antioxidant therapies in the setting of ischemic reperfused heart damage [49–51], that have been even

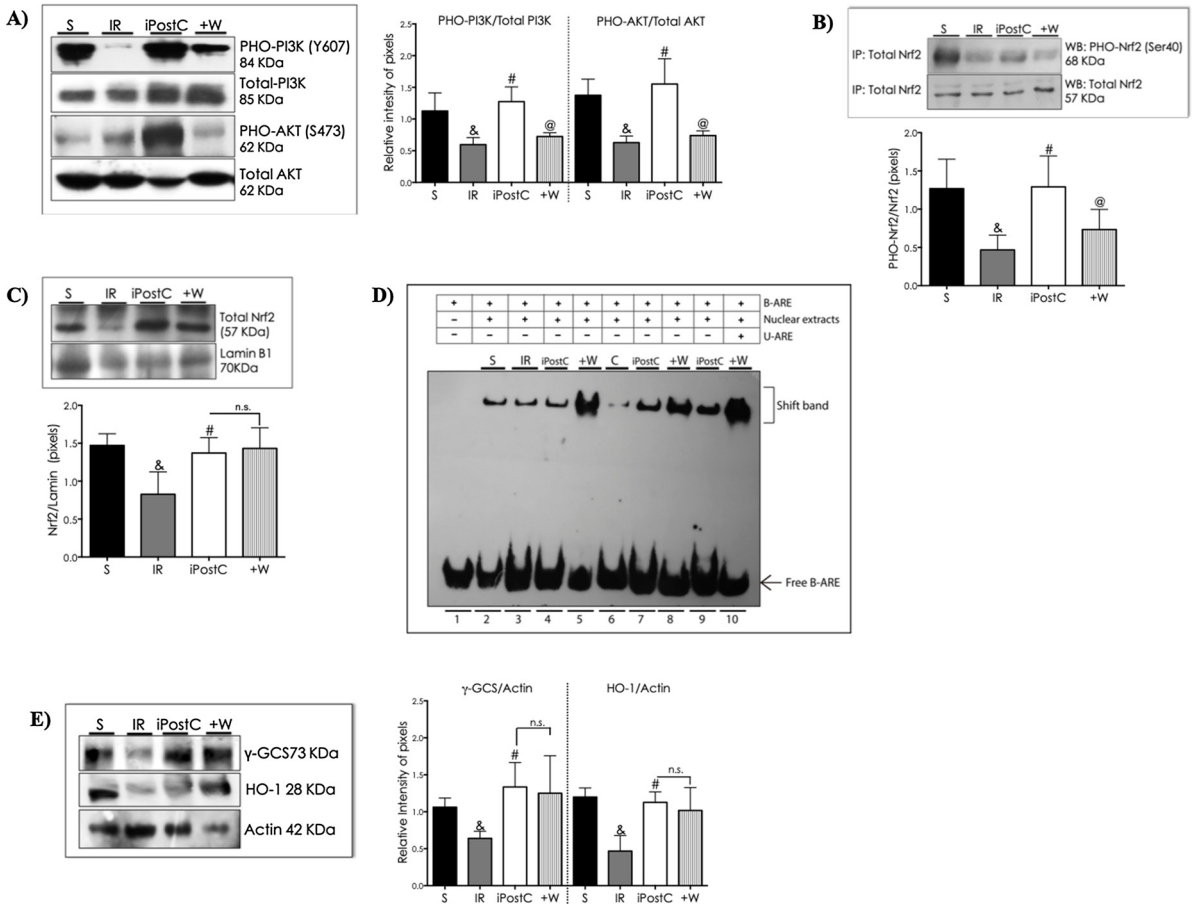

Fig. 7. Transcriptional activation of Nrf2 was not mediated by PI3K/AKT in the iPostC hearts.
(A) PHO-PI3K and PHO-AKT content obtained after 60 min of reperfusion related to their corresponding total protein. Sham (S), ischemic-reperfused (IR), ischemic post-conditioned (iPostC) and post-conditioned treated with wortmannin inhibitor (+W). (B) PHO-Nrf2 (Ser40) and total-Nrf2 detected in samples subjected to Nrf2:immunoprecipitation (IP). (C) Total Nrf2 content obtained after 60 min of reperfusion in nuclear fractions. Lamin B1 was evaluated as a loading marker. (D) EMSA showing free B-ARE migration (lane 1), complex formation in experimental groups (lanes 2–5 and 7–10) and competition assay (lane 6; C). (E) γ-Glutammil cysteine synthetase (γ-GCS) and hemeoxygenase-1 (HO-1) content after 60 min of reperfusion related with actin as loading marker. Data are the mean of at least 5 experiments ± S.D. $^{\&}p < .01$ vs. Sham; $^{\#}p < .01$ vs. IR; $^{@}p < .01$ vs. iPostC and non-significant (n.s.). (2-column fitting figure).

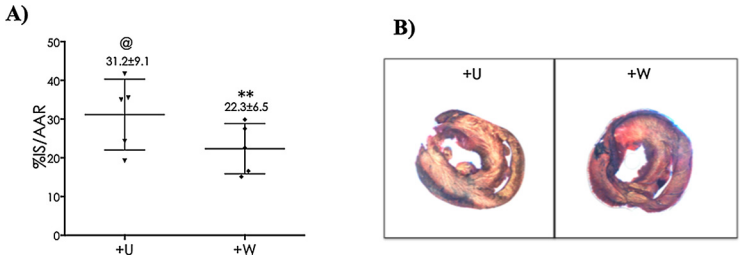

Fig. 8. Infarct size measurement.
A) Representative images of heart slices corresponding to inhibitors administration to iPostC hearts: +U (U0126) and + W (wortmannin) groups. Blue stain, non-risk area; Red stain, viable tissue; Pale stain, infarcted area. B) Infarct size was calculated as percentage related to area at risk. IS = Infarct size; AAR = area at risk; Data represents the mean of 5 independent experiments ± S.D. $^{@}p < .01$ vs. iPostC; $^{**}p < .05$ vs. iPostC. (1.5-column fitting column), color figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

deleterious [52], might result not only from inadequate delivering in the sites of ROS generation or from the inopportune concentration, but from the establishment of a more reductive state in the cardiac cell. We found that the impairment of heart performance by the *asc* effect in iPostC hearts correlated with hydrogen peroxide depletion and with the diminishment of PKCε and Erk1/2 activation but did not inhibit MEK and PI3K/AKT signaling in such conditions. In this sense, a prominent research group has proposed that ROS act as mediators rather than triggers of cardioprotection, as their concentration is raised after mitoKATP opening and regulate mitochondrial PKC activation [53]. According to our results we propose that hydrogen peroxide could act as a trigger of the redox signaling because its depletion by *asc* in the iPostC hearts, at the first minutes of reperfusion partially inhibited PKC and Erk1/2 activation. Although our results showed that PI3K/AKT activation was not redox-dependent in these conditions, it has been described that this pathway could be regulated by S-nitrosylation process. In ischemic brains PTEN (Phosphatase with sequence homology to tensin) was inhibited by S-nitrosylation augmenting AKT signaling promoting cell survival [54]. So far, many research groups have described the activation of the RISK pathway at early reperfusion to protect heart [13,14,55], nevertheless the targets involved by such activation have not been fully described.

The transcription factor Nrf2 contributes to maintain long-lasting protection in iPostC hearts, as it involves de novo synthesis of antioxidant proteins [21]. To evaluate the implications of redox signaling on downstream activation of Nrf2, we evaluated its activity after *asc* application in iPostC hearts and after pharmacological inhibition of ERK1/2 or PI3K, in order to know if Nrf2 could be a target of these

kinases. Our results show that Nrf2 activation is likely related with hydrogen peroxide-signaling since its depletion by *asc* in iPostC hearts decreased Nrf2 activation. Pharmacological inhibition of Erk1/2 did not inhibit Nrf2 transcriptional activation in iPostC hearts, neither phosphorylation at Ser40, nuclear accumulation nor binding to ARE sequence. Of note, phosphorylation of Ser40 in Nrf2 is crucial for its activation, promoting Keap1 disruption [56]. In the sequence of Nrf2 there are only 2 potential target sites for phosphorylation at Ser426 and Ser569 by Erk1/2, that is a Ser/Thr kinase, which phosphorylates preferentially Ser flanked by Pro residues [57]. Hypothetically, Nrf2 has not suitable residues to be phosphorylated by Erk1/2. Although, it has been reported that stimulating HepG2 cells with butilated hydroxyanisole, resulted in enhancement of Nrf2 activation mediated by Erk1/2 phosphorylation [58], nevertheless Nrf2 does not appear to be a direct target of Erk1/2 in the iPostC hearts, thus Erk1/2 signaling has other protective targets. In this context, our group reported that Erk1/2 is translocated into mitochondria of iPostC hearts contributing to preserve their function and conferring cell protection [59]. Therefore, the cardioprotective effects of Erk1/2 in iPostC hearts are independent of those exerted by Nrf2. Finally, pharmacological inhibition of PI3K in the iPostC hearts did not inhibit Nrf2 transcriptional activity, it would seem that the binding to ARE sequence was increased, although HO-1 and γ-GCS content were not changed in such conditions. For instance, there is a report that propose that PI3K/AKT/Nrf2 signaling confers cardioprotection in a model of hyperbaric oxygen preconditioning [60], however in the iPostC hearts the activation of Nrf2 is independent of PI3K signaling. Our results together indicate that Nrf2 activation in iPostC hearts is related with hydrogen peroxide-signaling in an-in-dependent way of the survival signaling of PI3K and Erk1/2.

Notably, a raising question would be whether this mechanism could be activated by iPostC also in female rat hearts, because IS has shown to be gender-dependent. Indeed, it has been reported that short-duration ischemia increases IS more in female than male rat hearts [61] and besides it seems that gender differences influence the activation of cardioprotective pathways [62,63].

5. Conclusion

We provide new insights about the paramount role of redox-balance preservation by iPostC and the role of hydrogen peroxide as a trigger of survival signals driven by kinase-dependent mechanisms. The cardiac protective signaling activated by iPostC include redox–dependent and –independent mechanisms, which act in concert activating different pathways in order to prevent reperfusion injury and concurs with the activation of Nrf2.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellsig.2019.109417.

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