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Diazoxide Postconditioning Induces Mitochondrial Protein S-Nitrosylation and a Redox-Sensitive Mitochondrial Phosphorylation/Translocation of RISK Elements: no Role for SAFE

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Abstract

Postconditioning (PostC) can be obtained either with brief cycles of ischemia/reperfusion (I-PostC) or with a direct targeting of mitochondria with Diazoxide (pharmacological PostC, P-PostC). I-PostC may induce the activation of RISK and SAFE pathways and may favor nitric oxide production with S-Nitrosylation of proteins and redox signaling. It is not clear whether Diazoxide can lead to similar effects. We compared the effects of I-PostC and P-PostC on *a)* kinases of RISK- and SAFE-pathway, *b)* S-Nitrosylation of mitochondrial proteins and *c)* reduction of death signals (PKCδ, cleaved-caspase3 and Beclin-1) in cytosolic and mitochondrial fractions. Isolated rat hearts, underwent *1)* perfusion without ischemia (Sham), *2)* ischemia/reperfusion (30-min ischemia plus 2-hours reperfusion), *3)* I-PostC (5 intermittent cycles of 10-s reperfusion and 10-s ischemia immediately after the 30-min ischemia), *4)* P-PostC (Diazoxide 30 μM in the first of 3-min of reperfusion) or *5)* I-PostC+MPG or P-PostC+MPG (MPG, 2-mercaptopropionylglycine 300 μM). Using Western blot and biotin switch assay, we found that P-PostC induced a redox sensible phosphorylation/translocation of Akt, ERK1/2 and GSK3β into the mitochondria, but not of phospho-STAT3, which was translocated into the mitochondria by I-PostC only. Either I-PostC or P-PostC increased mitochondrial S-Nitrosylated proteins (*e.g.*, VDAC) and reduced the levels of phospho-PKCδ, cleaved-caspase3 and Beclin-1. Therefore, direct targeting of mitochondria with Diazoxide *a)* activates the RISK-pathway *via* a redox signaling, *b)* favors discrete mitochondrial protein S-Nitrosylation, including VDAC and *c)* decreases signals of death. Intriguingly, phospho-STAT3 translocation is induced by I-PostC, but not by P-PostC, thus suggesting a redox-independent mechanism in the SAFE-pathway.

Key words: Cardioprotection; Ischemia; Nitric oxide; Reperfusion; RISK; SAFE.

Introduction

Reperfusion is the only way to salvage an ischemic organ, but it is a double-edged sword and can induce severe and irreversible damages to the myocardium, collectively referred to as *reperfusion injury* [42,76,88]. Cycles of brief reperfusion and ischemia performed immediately at the onset of reperfusion following a prolonged ischemic insult markedly limits reperfusion injury. This cardioprotective phenomenon is named *Ischemic Postconditioning* (I-PostC), which is as powerful as ischemic preconditioning [89]. I-PostC may limit all forms of cell death induced by ischemia and reperfusion (I/R) [20,21,41,44,72].

It is known that I-PostC induces the activation of Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways [33,34,40,75], with a potential dependence of signaling on species and models [77,78]. While the SAFE pathway recognizes STAT3 activation as a central point, the RISK pathway includes the activation/phosphorylation of several kinases (*e.g.*, Akt, ERK1/2, GSK3β) [33,34,79]. Nevertheless, both pathways converge on mitochondria influencing their function. In particular, in the mitochondria, cardioprotective pathways lead to the opening of mitochondrial K_{ATP} dependent (mitoK_{ATP}) and closure of mitochondrial transition pore (mPTP) with consequent integrity of these organelles [9,35,67,70]. mitoK_{ATP} channel activation may be involved in the production of small quantity of reactive oxygen species (ROS), which helps to preserve integrity and proper functioning of mitochondria themselves [1,14,24,62,66,70]. In fact, like preconditioning, PostC depends on ROS signaling [25,69,84]. The I-PostC maneuvers also induce the production of nitric oxide ($NO^{\cdot}$) and reactive nitrogen species (RNS) [69], which can lead to mitochondrial proteins S-Nitrosylation (SNO) [25,68,80-83]. Then ROS/RNS in turn lead to the activation of survival kinases such as MAP kinases, tyrosine kinases and PKCs, including PKCε and PKCδ, which may have opposing roles [12,15,16,31,36,45,58,62].

Therefore cardioprotective stimuli starting from outside the mitochondria converge on these organelles and then reverberate in the cytosol again. Specifically, I-PostC leads to the translocation/phosphorylation of effectors of the RISK and SAFE pathways to the mitochondria, then signals from mitochondria affects cytosolic elements again [7,19,33,34,62,66].

A protection similar to that obtained with I-PostC has been obtained with pharmacological agents given in early reperfusion, referred to as *Pharmacological PostC* (P-PostC). Many of these pharmacological treatments target directly mitochondria [17,33,46]. Recently, we have demonstrated that the infusion of *Diazoxide*, a direct and selective opener of mitoK_{ATP} channels, induces cardioprotection when infused in the initial three min of reperfusion [65]. Diazoxide mediated cardioprotection requires a ROS signaling, both in ischemic pre- and post-conditioning [27,65]. Most notable, preconditioning with Diazoxide can increase $NO^{\cdot}$ bioavailability [4] and protein translocation [4,27,51].

Therefore, we hypothesized that P-PostC with Diazoxide includes SNO-proteins and a ROS signaling that triggers a cross talk between mitochondria and cytosol, with consequent translocation of cardioprotective factors from cytosol to mitochondria.

Also when the ischemic heart is reperfused in the absence of any cardioprotective maneuvers, a cross-talk between cytosol and mitochondria is responsible for the activation of several mechanisms which favor the development of "*signals of death*" leading to oncosis, apoptosis and/or autophagy [54,64,66]. Among these, a role is played by PKCδ translocation within mitochondria, where it induces several metabolic modifications, increases mitochondrial ROS-stress, and promotes apoptosis [16]. Importantly, both I-PostC and some P-PostC reduce some of these *signals of death* [67,81]. To the best of our knowledge there are no studies on the effects of P-PostC with Diazoxide on signals of death.

Our study was aimed to compare the effects of two different types of postconditioning (I-PostC and P-PostC, which target *indirectly* and *directly* mitochondria, respectively) in terms of *1)* kinase phosphorylation and translocation, *2)* amount of nitrosylated proteins, and *3)* modification of "*signals of death*" in mitochondrial fractions obtained from reperfused hearts.

Materials and Methods

Animals

Male Wistar rats (5-6 month old, body weight 450–550 g) were purchased from Janvier (Le Genest-St-Isle, France). Animals received humane care in compliance with Italian law (DL-116, Jan. 27, 1992) and in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Perfusion technique

Methods used were similar to those previously described [61,63,65,69]. In brief, isolated rat hearts were retrogradely perfused at constant flow (9±1 ml/min/g) with oxygenated Krebs–Henseleit buffer (127 mM NaCl, 17.7 mM $NaHCO_3$, 5.1 mM KCl, 1.5 mM $CaCl_2$, 1.26 mM $MgCl_2$, 11 mM D-glucose (Sigma-Aldrich, St. Louis, MO, USA) gassed with 95% O_2 and 5% CO_2 [61,63,65,69]. Hearts were paced at 280 bpm and kept in a temperature-controlled chamber (37°C). Coronary perfusion pressure and left ventricular pressure were monitored to assess the preparation conditions.

Experimental protocols

Infarct size groups

After the stabilization period, hearts were randomly divided in three groups (n=6 for each group): 1) I/R group, hearts underwent 30-min of global ischemia and then a period of 120-min full reperfusion; 2) I-PostC group, after 30-min ischemia, hearts underwent a PostC protocol (5 cycles of 10-s reperfusion and 10-s global ischemia) then a period of 120-min full reperfusion [63,65,69]; 3) P-PostC group, after 30-min ischemia, hearts were infused with Diazoxide (30 μM) [27,32,65] for 3-min and then underwent a period of 120-min full reperfusion.

In two additional groups of hearts (P-PostC+MPG and I-PostC+MPG; n= 5 for each group), the mitochondrial specific antioxidant, 2-mercaptopropionylglycine (MPG, 300 μM), was co-infused

with Diazoxide (30 μM) or during I-PostC maneuvers, in the initial 3-min of 120-min reperfusion, in order to verify the role of redox signaling [27,65 and references therein].

Assessment of myocardial injury

Infarct areas were assessed at the end of the experiments with the nitro-blu-tetrazolium technique (Sigma-Aldrich, St. Louis, MO, USA) in a blinded fashion, as previously described [61,63,65,69]. In brief, immediately after reperfusion hearts were rapidly removed from the perfusion apparatus and the left ventricle dissected into 2–3 mm circumferential slices. Following 20-min of incubation at 37°C in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer, who was unaware of the protocols. The necrotic mass was expressed as a percentage of risk area (*i.e.,* total left ventricular mass).

Western blotting and S-Nitrosylation

Isolation of rat heart mitochondria

Seven additional groups of hearts were used to separate the mitochondrial and cytosolic fractions: five experimental groups (n= 6 for each Group) are similar to those used for the evaluation of infarct size; the other two groups are *1)* a Sham (non-ischemic) group in which hearts underwent 80-min buffer-perfusion only (n=6; Sham group) and *2)* a non-ischemic group, in which, for comparative purpose, Diazoxide (DZO) was infused for 3-min after the stabilization period (n= 4; Sham+DZO group). Mitochondrial and cytosolic fractions were obtained by a modification of published procedures [3,10]. In brief, the left ventricle was quickly submerged in 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, and 0.1% (w/v) BSA (pH 7.4). The tissue was finely minced with scissors and then homogenized in the same buffer (10 ml buffer per g tissue), using an ice-cold Potter. Large cell debris (membrane and nuclear fractions) were pelleted by centrifuging the homogenate twice for 5-min at 600 g and discarded avoiding solubilization of

membrane proteins. Mitochondria were pelleted by 10-min centrifugation at 10,300 g. The supernatant was centrifuged for 1 h at 100,000 g to obtain cytosolic fraction. The mitochondrial pellet was suspended, loaded in tubes containing 30% (v/v) Percoll in 225 mM mannitol, 1 mM EGTA, 25 mM HEPES, 0.1% (w/v) BSA (pH 7.4) and spun for 30-min at 95,000 g in a Beckman Coulter centrifuge with SW40 rotor. Mitochondria were collected from the lower part of the dense, brownish yellow mitochondrial band [67]. Protein level in each fraction was determined by Bradford's method [11].

Western blotting

About 50 μg of protein extracts were separated by SDS-PAGE on 8% [for Na^+/K^+ ATPase, PKCδ, phospho-PKCδ, STAT3, phospho-STAT3, vinculin, mitochondrial trifunctional enzyme α subunit (HADHA)] 10% [for HSP60, voltage-dependent anion-selective channel (VDAC), protein disulfide isomerase (PDI), Lamin A/C, Akt, phospho-Akt, GSK3β, phospho-GSK3β, Beclin-1] or 12% [for Phospholamban (PLN) and cleaved caspase-3] gels and transferred to PVDF membrane (GE Healthcare, Buckinghamshire, UK). The membranes were incubated overnight with the following primary antibodies: anti- Na^+/K^+ ATPase α1 (Upstate Biotechnology, Billerica, MA, USA), anti ERK1/2, anti-phospho-(Thr-202/Tyr-204)-ERK1/2; anti-PKCδ, anti-phospho-(Ser-643)-PKCδ, anti-Akt, anti-pospho-(Ser-473)-Akt, anti-GSK3β, anti-phospho-(Ser-9)-GSK3β, anti-cleaved-caspase-3, anti-phospho-(Ser-727)-STAT3, anti-phospho-(Tyr-705)-STAT3 (Cell Signaling Technology, Beverly, MA, USA), anti-Beclin-1 (Sigma-Aldrich, St. Louis, MO, USA), anti-HSP60 (BD Biosciences, Franklin Lakes, NJ, USA), anti-Lamin A/C and anti-PLN (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-VDAC and anti-PDI (Abcam, Cambridge, UK) all were diluted according to the manufacturer's instructions. Immunoblotted proteins were visualized by using Immuno-Star HRP Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by Kodak Image Station 440CF. To confirm equal protein loading, blots were

stripped with 0.4 M NaOH and then re-blotted for all antibodies as well as for anti-vinculin antibody (Sigma-Aldrich, St. Louis, MO, USA) or for anti-HADHA (Abcam, Cambridge, UK) for cytosolic and mitochondrial fractions, respectively. Image analyses were performed by the Kodak 1D 3.5 software [67].

Biotin switch assay for the detection of S-Nitrosylated proteins

All preparative procedures were performed in the dark (covering all equipments with aluminum foil) to prevent light-induced cleavage of nitrosylations [2,47,68,82,86]. Mitochondrial fractions were incubated and centrifuged on ice in appropriate buffers (20 mmol l^{-1} Tris pH 7.5, 150 mmol l^{-1} NaCl, 1% Igepal CA 630, 0.5% Sodium Deoxycholate, 1 mmol l^{-1} EDTA, 0.1% SDS, 200 mmol l^{-1} Sodium Orthovanadate, and Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). Proteins were quantified with Bradford's method [11], and the biotin switch assay with or without ascorbate (1mM) was performed as previously described [2,47,68]. In particular, blocking buffer (225 mmol l^{-1} HEPES, pH 7.7, 0.9 mmol l^{-1} EDTA, 0.09 mmol l^{-1} neocuproine, 2.5% SDS, and 20 mmol l^{-1} MMTS) and HENS buffer (250 mmol l^{-1} HEPES, pH 7.7, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} neocuproine, and 1% SDS) were used (Sigma-Aldrich, St. Louis, MO, USA) [2,47,68].

To detect biotinylated proteins by Western blot, samples from the biotin switch assay were separated on 12% SDS-PAGE gels, transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK), blocked with non-fat dried milk (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and incubated with streptavidin-peroxidase, diluted 1/10000 for 1 h. To confirm equal protein loading, blots were stripped with 0.4 M NaOH and then re-blotted with an anti-vinculin antibody (Sigma-Aldrich, St. Louis, MO, USA). Image analyses were performed by the Kodak 1D 3.5 software [67,68]. Since VDAC has a cysteine residue that can be nitrosylated

[4,57], we assessed VDAC S-Nitrosylation, using the SNO specific protein method adopted by several authors [2,26,60,75,82,86], including us [68].

.Statistical analysis

All values are expressed as mean±SE. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post test. p Value≤0.05 was considered to be significant.

Results

Infarct size (Fig 1)

Total infarct size, expressed as a percentage of left ventricular mass, was 63±2% in I/R Group. In I-PostC, infarct size was significantly lower, 29±2% ($p<0.01$ *vs* I/R group). Similar results were obtained with P-PostC (Diazoxide infusion), with a significant reduction of infarct size with respect to I/R group (34±6%, $p<0.05$ *vs* I/R; NS, not significant *vs* I-PostC). As previously reported [18,65,84], the protective effects of I-PostC or P-PostC (Diazoxide infusion) were abolished by the infusion of the antioxidant MPG at the beginning of reperfusion.

Western blotting

Marker/protein levels were studied by Western-blot analysis in cytosolic and mitochondrial fractions. Representative bands and densitometric analysis of the scanned blots are presented in all the following figures. Data are normalized with respect to the mean value of the Sham group, including the raw data of Sham.

The integrity and purity of mitochondrial fraction (Fig 2)

To study mitochondrial integrity we assessed the levels of HSP60 (Fig 2A) [30]; to study the purity of mitochondrial (MF) we verified the presence of Na^+/K^+ ATPase, VDAC, Lamin A/C, PLN, PDI and vinculin (Fig 2B) [6,9,13,46,53,60,71], in both MF and cytosolic fraction (CF) extracted from non-ischemic (Sham), post-ischemic (I/R) and postconditioned (I-PostC and P-PostC) hearts.

As expected, levels of HSP60 were lower in CF than in MF in the Sham group (see bands in Fig 2A). However, the densitometry revealed that in I/R group the levels of HSP60 decreased in the MF and increased in the CF ($p<0.05$ *vs* all other groups for both fractions, Fig 2A). Since both I-PostC and P-PostC maneuvers kept HSP60 levels similar to those of Sham group in both CF and

MF, it may be inferred that these maneuvers preserved mitochondrial integrity. These results are in agreement with those obtained in our previous study [67].

As expected, VDAC, a typical protein of mitochondrial membrane, was found in the MF and was undetectable in the CF. Yet, the levels of Na^+/K^+ ATPase, PLN and Lamin A/C, typical proteins of cellular membrane, sarcoplasmatic reticulum and nucleus, respectively, were undetectable in the MF and present in the remaining fraction, in all experimental groups. Together, these results suggest that our MF was substantially free of contaminants.

However, PDI and vinculin, which are considered typical of the endoplasmic reticulum and cytoskeleton, respectively, are present in both CF and MF. These findings are in agreement with the observations of several authors for endoplasmic proteins, including PDI [48,53], and for cytoskeletal proteins, such as tubulin and vinculin [13,60]. Nevertheless, the absence of nuclear proteins in the MF is a prerequisite for the assessment of STAT3 in the SAFE pathway [9,34,49,79].

P-PostC induced a redox dependent translocation to mitochondria of phosphorylated kinases of the RISK pathway (Figs 3-5)

Akt, ERK1/2 and GSK3β are important cardioprotective kinases of the RISK pathway in postconditioning [33,34,79]. Here we analyzed the role of ROS signaling in P-PostC induced mitochondrial translocation of these kinases.

Levels of Akt are reported in Fig 3. Of note, an elevated phospho/total ratio was observed in the two protected groups ($p<0.05$ *vs* Sham and I/R groups for both PostC protocols). Moreover, the ROS scavenger, MPG, avoided P-PostC-induced Akt phosphorylation in mitochondrial fraction.

As can be seen in Fig 4, also the levels of phospho-ERK1/2 are significantly higher in the two protected groups ($p<0.05$ *vs* Sham and I/R Group for both PostC protocols). Again, MPG avoided P-PostC-induced ERK1/2 phosphorylation within mitochondria.

Levels of GSK3β are reported in Fig 5. The phospho/total ratio of GSK3β resulted significantly lower in I/R group ($p<0.05$ *vs* Sham), while was significantly higher in the two protected groups ($p<0.05$ *vs* Sham and I/R Group for the two PostC protocols). Yet, the antioxidant, MPG, limited P-PostC-induced GSK3β phosphorylation in mitochondrial fraction.

As expected, phosphorylated forms of Akt, ERK1/2 and GSK3β were increased in the cytosolic fraction by both PostC protocols (data not shown).

SAFE pathways: only I-PostC induced a significant increase of phospho-STAT3 within mitochondria (Fig 6).

STAT3 is one of the major players of the protective SAFE pathway [9,34,43] and it has been reported that in protected hearts its phosphorylation is necessary for a mitochondrial localization [34,85]. Therefore, to evaluate the activation of the SAFE pathway, phosphorylation of STAT3 on both Ser727and Tyr705 were studied within mitochondria (Fig 6). While I-PostC induced a significant increase of both phospho-STAT3 within mitochondria ($p< 0.005$ *vs* all other groups), P-PostC with Diazoxide was unable to induce a similar effect.

I-PostC and P-PostC can increase the S-Nitrosylation of mitochondrial proteins (Fig 7)

Nitric oxide has been shown to be an important mediator of cardioprotection and the addition of an $NO^{\cdot}$ group to a protein thiol is known as S-Nitrosylation (SNO) [62]. As can be seen in Fig 7, either I-PostC or P-PostC with Diazoxide increased SNO proteins total levels with respect to I/R group. Diazoxide given to Sham hearts induced the highest level of SNO proteins, including VDAC S-Nitrosylation. Importantly, the levels of nitrosylated VDAC are higher in postconditioned hearts than in Sham and I/R groups.

I-PostC and P-PostC could reduce the "signals of death"(Fig 8)

As expected, I/R significantly ($p<0.01$) raised all the signals of death with respect to Sham. The two cardioprotective protocols limited these increases, and maintained Sham levels for all the

enzymes (Fig 8 panels A,C,D), with the exception of mitochondrial PKCδ levels (Fig 8, panel B), which were also reduced by the two protective protocols, but not to Sham level by P-PostC ($p < 0.05$ P-PostC *vs* Sham).

Discussion

Here we demonstrate, for the first time, that P-PostC cardioprotection with Diazoxide leads to the phosphorylation and translocation of kinases of RISK pathway to the mitochondria. This translocation is similar to what seen for preconditioning by many authors for both Diazoxide- and ischemic-preconditioning [4,32,56,75]. As far as we know, kinase translocation within mitochondria after I-PostC has been demonstrated by few authors [7,10]. Here we also demonstrate that I-PostC and P-PostC with Diazoxide induce a similar increase in S-Nitrosylation of mitochondrial proteins. Among these proteins, we detected S-Nitrosylation of VDAC, which, in fact, has a cysteine residue that can be nitrosylated and may play a critical role in the mitochondrial permeability transition pore formation [4,35,38,57,60].

However, differently from I-PostC, P-PostC does not induce STAT3 mitochondrial phosphorylation. Since MPG avoids translocation of RISK elements induced by P-PostC, we suggest that the redox signaling activated by Diazoxide affects RISK- but not SAFE-pathway.

Finally, either I-PostC or P-PostC reduces the activation of *signals of death* caused by I/R.

S-Nitrosylation (SNO): Nitric oxide is reported to increase SNO of different mitochondrial targets [29]. Protein SNO is a redox-reversible posttranslational protein modification which may play an important cardioprotective role in PostC maneuvers. For example, $NO^{\cdot}$ increases SNO of complex I thereby reducing (not abolishing) ROS generation. SNO also inhibits complex V and blocks irreversible oxidation of proteins [29,62]. In addition to I-PostC, also estrogen-induced increase in contractility has been shown to involve an increase of SNO of several proteins and an upregulation of cardiac eNOS and nNOS [82].

Importantly, RISK activation in PostC results in NOS activation [33,34,79] thereby explaining increased SNO of proteins by I-PostC and indirectly by P-PostC with Diazoxide. Intriguingly, the SNO of mitochondrial proteins, including VDAC, is particularly evident when Diazoxide is given

to non-ischemic hearts (Fig 7); thus suggesting that Diazoxide increases NO formation and favors S-Nitrosylation. Actually, it has been reported that Diazoxide increases the levels of dimethylarginine dimethylaminohydrolase (DDAH) [51]. Since DDAH metabolizes asymmetric dimethylarginine (an endogenous NOS inhibitor), Diazoxide might increase $NO^{\cdot}$ availability and SNO *via* the intervention of DDAH [23]. This deserves to be further studied.

RISK and SAFE pathways: Protective signaling molecules of RISK and SAFE are activated in response to stress to prevent cell death [33,34,79]. Our present results demonstrate that redox-dependent signals can start from mitochondria (Diazoxide effect) to modulate RISK, but not SAFE elements. Previous studies have suggested an essential role for ROS signaling in PostC protection [25,69,84], but, to the best of our knowledge, no one has studied the role of RISK and SAFE in P-PostC with Diazoxide, a drug supposed to promote ROS signaling through actions on mitoK_{ATP} channels, with the contribution of mitochondrial connexin-43 [7,10,37,71,73].

RISK: Akt and ERK1/2 are reported to phosphorylate and inhibit GSK3β resulting in cardioprotection *via* multiple targets within mitochondria [33,34].

Akt can translocate to subcellular compartments including the nucleus and mitochondria [55,74]. There is a correlation between Akt accumulation in mitochondria and phosphorylation/inhibition of mitochondrial GSK3β in these organelles [5]. Yet, GSK3β prefers substrates that have been previously phosphorylated by another kinase. This preference for primed substrates provides a mean to integrate multiple signaling pathways within mitochondria. Although the role of GSK3β seems controversial, the majority of data suggests an important role for the phosphorylation of this enzyme in a cardioprotective scenario; for a review see [80].

The mitochondrial translocation of phospho-Akt is an important signal for cardioprotection and it is observed in different models of apoptosis prevention, including neuronal and cardiac cells, where activated Akt, besides GSK3β, may phosphorylate other mitochondrial proteins

[1,5,50,55]. The importance of ERK1/2 in reperfusion in pre- and post-conditioning has been confirmed several times. For instance, Fryer *et al.* [28] reported rapid activation of ERK1/2 after pre-conditioning and then a second activation at 5 minutes of reperfusion, which was maintained after 30 and 60 minutes of reperfusion in the cytosolic and nuclear fractions. Yang *et al.* [87] and Darling *et al.* [22] reported that postconditioning resulted in an increase in phospho-ERK1/2 and consequent heart protection, which were abrogated by an inhibitor of ERK1/2. Although the activity of Akt in mitochondria is not well defined, the phosphorylation/activation of Akt is a pivotal event to induce cardioprotection. Accordingly, in I-PostC and in P-PostC phospho-Akt is significantly increased in the mitochondrial fraction (Fig 3). Moreover, here we report an increased mitochondria localization of phospho-ERK1/2 following I-PostC and P-PostC (Fig 4). While GSK3β phosphorylation/deactivation is reduced in I/R, its phosphorylation is similarly increased in the two cardioprotective protocols (Fig 5). Importantly, MPG reduces the levels of the phosphorylated forms of the three RISK elements induced by P-PostC in MF (Figs 3, 4 and 5), thus supporting an intriguing role of the ROS-signaling in the activation/translocation of RISK by P-PostC. This is in line with the ROS signaling-dependent phosphorylation of RISK elements induced by I-PostC, with or without mitochondrial translocation of kinases [7,10,17,32,62].

SAFE: in I-PostC cardioprotection enhanced STAT3-phosphorylation and improved mitochondrial function have been confirmed in several species including pigs *in vivo* and *in vitro* [39,49]. The action of STAT3 on cardiac mitochondria and in particular on the inhibition of mPTP opening has been recently studied by Boengler *et al.* [8]. We confirm the presence of both Ser- and Tyr-phosphorylated STAT3 after I-PostC within mitochondria; however the lack of increase of phospho-STAT3 after the P-PostC with Diazoxide suggests that the SAFE pathway is a parallel pathway of RISK in I-PostC only. These two pathway can be activated simultaneously (I-PostC) and, perhaps, may cross-talk [34]. However, the SAFE pathway seems not necessary. In

fact, the direct targeting of mitochondria by Diazoxide is protective, even though does not activate SAFE pathway (*i.e.*, Diazoxide does not reverse the tendency in phospho-STAT3 reduction induced by I/R). This is in line with the observation that mice lacking cardiac STAT3 are still protected by I-PostC [34], though the number of postconditioning cycles is a critical factor to be considered for the successful effect of I-PostC in both aged and STAT3-deficient hearts [6]. Besides STAT3, also STAT5 activation has a cardioprotective role in humans [40] and the possibility of a vicariant protective effect of these two STATs deserves to be further studied.

Signals of death: Importantly, either I-PostC or P-PostC maneuvers reduce the activation of *signals of death* caused by I/R. Both protocols, in fact, similarly preserve mitochondria integrity, as demonstrated by HSP60. Moreover, they also reduce cleaved caspase3, Beclin-1 and phosphorylation/translocation of PKCδ, which are implicated in all forms of cell death (*apoptosis, autophagy and necrosis*). Of note, it has been reported that increases in autophagic activity in the reperfusion phase are accompanied by upregulation of Beclin-1 in the heart [54]. Here we demonstrate that mitochondrial Beclin-1 is increased in I/R and that this increase is prevented by either I-PostC or P-PostC. However, the signaling mechanism by which I/R upregulates and PostC downregulates Beclin-1 expression is unknown. Notably, PKCδ may be a double-edged sword, though its phosphorylation more consistently triggers cell death [15,31,45,52,58]. Here we observe that I-PostC is more effective than P-PostC in reducing the phospho-PKCδ/total PKCδ ratio. Whether this difference is due to the lack of SAFE pathway activation in P-PostC needs to be determined.

Mitochondrial translocation of proteins and limitation of the study: In all cells an important trafficking across cytosol and mitochondria is present. However, the mechanisms for kinase trafficking is only partially known [12,59]. Heat shock proteins and potential across the mitochondrial inner membrane may play an important role in mitochondrial import of specific

protein [5,12]. Future studies should investigate the mechanisms of different trafficking in I-PostC and P-PostC. In a recent review of Gucek & Murphy [29] it is reported that: "Elucidating the role of the mitochondria in cardioprotection will require a combination of examining candidate proteins as well as unbiased proteomic studies. The large-scale proteomics approach with the use of fractionation/enrichment is useful to identify unexpected protein changes. However, for low abundance signalling molecules, a targeted approach is necessary". Here we report data collected with a "targeted approach". Future proteomic and systems biology studies may analyze the mechanism of different mitochondrial protein import in cardioprotection.

In conclusion, here we show the redox-sensitive localization of many phosphorylated RISK kinases into the mitochondria following both "classic" I-PostC and P-PostC with Diazoxide. However, while I-PostC also increased phosho-STAT3 within mitochondria, P-PostC does not. This suggests that signals upstream of mitochondria are required to activate SAFE pathway leading to STAT3 phosphorylation and subsequent mitochondrial localization [39]. Yet during the two cardioprotective protocols we observe an increased S-Nitrosylation of mitochondrial proteins, such as VDAC, which is considered an important signal of cardioprotection. In fact, both protocols down-regulate molecules, which can induce the death of cardiac cells.

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Figure Legends

Figure 1. Infarct size (IS).

The amount of necrotic tissue is expressed as percent of the left ventricle (LV), which is considered the risk area. I/R: ischemia/reperfusion; I-PostC: ischemic postconditioning; P-PostC: pharmacological postconditioning with Diazoxide; I-PostC+MPG: the antioxidant MPG was given during I-PostC maneuvers; P-PostC+MPG: MPG was co-infused with Diazoxide. **$p < 0.01$ and *$p < 0.05$ Vs. I/R. NS: not significant.

Figure 2. Integrity and Purity of Isolated Mitochondria.

A) Levels of HSP60 are used as indices of the integrity of mitochondria in non-ischemic (Sham), post-ischemic (I/R) and postconditioned (I-PostC and P-PostC) groups. Mean±SE of HSP60 levels in mitochondrial and cytosolic as well as HSP60 representative blots are reported. Data are expressed in arbitrary units (a.u.) and normalized for loading controls. *$p<0.05$ vs Sham.

B) The presence of Na+/K+ ATPase, voltage-dependent anion-selective channel (VDAC), protein disulfide isomerase (PDI), Lamin A/C, Phospholamban (PLN) and Vinculin is used as index of the purity of mitochondria in Sham, I/R, I-PostC and P-PostC groups. Representative blots of bands from mitochondrial and cytosolic fractions are reported.

Figure 3. Mitochondrial Akt.

Levels of total and phosphorylated Akt (phospho-Akt) have been analyzed in Sham, I/R, I-PostC, P-PostC, Sham+MPG and P-PostC+MPG groups. Mean±SE of the relative phospho-Akt/total Akt ratio in mitochondrial fraction and their representative blots are reported. Data are normalized for loading control (HADHA) and then with respect to mean value of Sham group. *p<0.05 vs Sham; # p< 0.05 *vs* I/R. NS: not significant.

Figure 4 Mitochondrial ERK1/2.

Levels of total and phosphorylated ERK1/2 (phospho-ERK1/2) have been analyzed in Sham, I/R, I-PostC, P-PostC groups, Sham+MPG and P-PostC+MPG groups. Mean±SE of the relative phospho-ERK1/2/total ERK1/2 ratio in mitochondrial fraction and their representative blots are reported. Data are normalized for loading control (HADHA) and then with respect to mean value of Sham group. *p<0.05 *vs* Sham; # p< 0.05 *vs* I/R. NS: not significant.

Figure 5. Mitochondrial GSK3β.

Levels of total and phosphorylated GSK3β (phospho- GSK3β) have been analyzed in Sham, I/R, I-PostC, P-PostC, Sham+MPG and P-PostC+MPG groups. Mean±SE of the relative phospho-GSK3β/total GSK3β ratio in mitochondrial fraction and their representative blots are reported. Data are normalized for loading control (HADHA) and then with respect to mean value of Sham group. *p<0.05 *vs* Sham; # p< 0.05 *vs* I/R. NS: not significant.

Figure 6. Mitochondrial p-Ser-STAT3.

Levels of total and phosphorylated in Ser-STAT3 (p-Ser-STAT3) and in Tyr-STAT3 (p-Tyr-STAT3) have been analyzed in I/R, I-PostC and P-PostC groups. Mean±SE of the relative phospho-STAT3/total STAT3 ratio (for both sites of phosphorylation) in mitochondrial fraction and their representative blots are reported. Data are normalized for loading control (HADHA) and then with respect to mean value of Sham group. **$p<0.01$ *vs* Sham; ## $p< 0.05$ *vs* I/R.

Figure 7. S-Nitrosylation (SNO) of Mitochondrial Proteins.

Levels of total SNO proteins have been analyzed by biotin switch assay in the mitochondrial fraction of the Sham, Sham+DZO, I/R, I-PostC and P-PostC groups. To verify the specificity of the biotin switch assay, ascorbate has been omitted in Sham samples (-Asc). The analysis of total bands shows an increase in S-Nitrosylation in I-PostC and P-PostC as well as in Sham+DZO. Mean±SE of the levels of SNO proteins in mitochondrial fraction and their representative blots are reported. Data are reported as normalized arbitrary units (a.u.). Specific protein analysis shows an increased SNO of VDAC in I-PostC, P-PostC and in Sham+DZO. *$p<0.05$ *vs* Sham; # $p< 0.05$ *vs* I/R. **$p<0.05$ *vs* Sham; ## $p< 0.05$ *vs* I/R. NS: not significant.

Figure 8. Death Signals.

A and B) Cytosolic and Mitochondrial PKCδ. Levels of total and phosphorylated PKCδ (p-PKCδ) have been analyzed in non-ischemic (Sham), post-ischemic (I/R) and postconditioned

(I-PostC and P-PostC) groups in both in cytosolic and mitochondrial fractions. Presented bands were not juxtaposed in the original film. Data are normalized for loading control (vinculin or HADHA) and then with respect to mean value of Sham group. Mean±SE of the relative phospho-PKCδ/total PKCδ ratio in mitochondrial and cytosolic fraction and their representative blots are reported. **$p<0.01$ *vs* all other groups; # $p< 0.05$ *vs* I/R.

C) Cytosolic cleaved-caspase-3. Levels of cleaved-caspase-3 have been analyzed in the cytosolic fraction of the four experimental groups (Sham, I/R, I-PostC and P-PostC groups). Data are normalized for loading control (vinculin) and then with respect to mean value of Sham group. Mean±SE in cytosolic fraction and their representative blots are reported. **$p<0.01$ *vs* all other groups

D) Mitochondrial Beclin-1. Levels of Beclin-1 have been analyzed in the mitochondrial fraction of the four experimental groups (Sham, I/R, I-PostC and P-PostC groups). Data are normalized for loading control (HADHA) and then with respect to mean value of Sham group. Mean±SE in cytosolic fraction and their representative blots are reported. **$p<0.01$ *vs* all other groups.

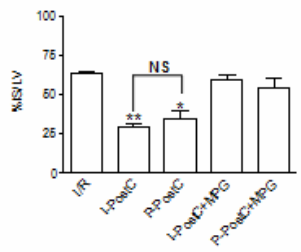
100
75
50
25
0
%ESLV
NS
**
*
I/R
I-PostC
P-PostC
I-PostC+MPG
P-PostC+MPG

Fig.1

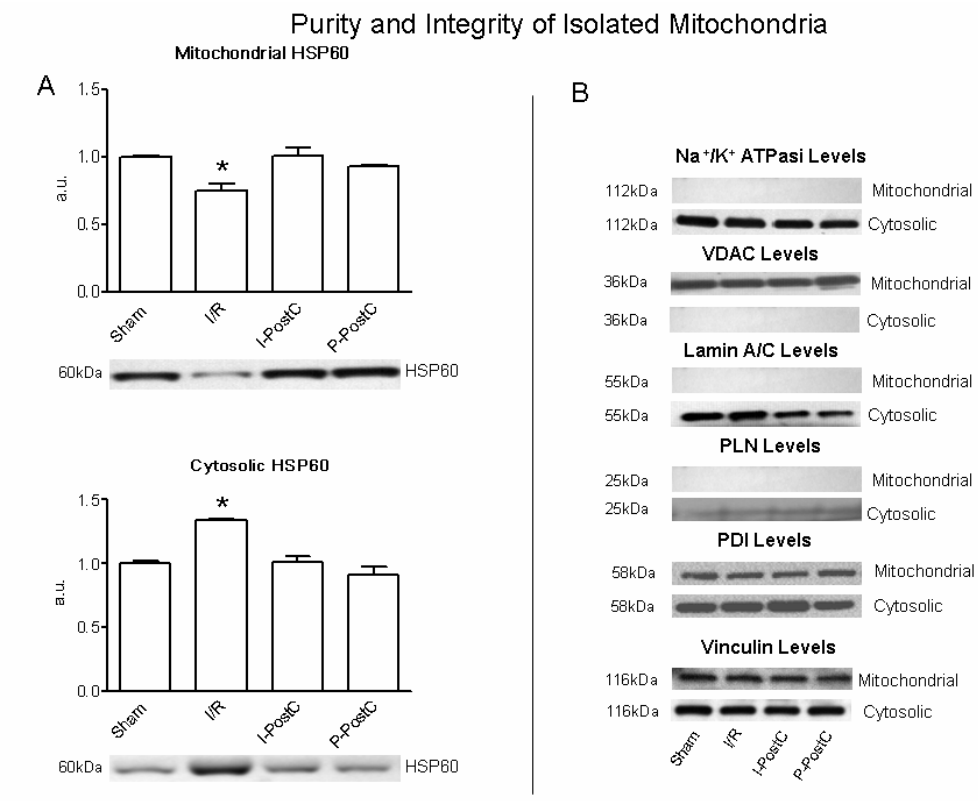
Purity and Integrity of Isolated Mitochondria
Mitochondrial HSP60
A
1.5
1.0
0.5
0.0
a.u.
*
Sham
I/R
I-PostC
P-PostC
60kDa
HSP60
Cytosolic HSP60
1.5
1.0
0.5
0.0
a.u.
*
Sham
I/R
I-PostC
P-PostC
60kDa
HSP60
B
Na+/K+ ATPasi Levels
112kDa
Mitochondrial
112kDa
Cytosolic
VDAC Levels
36kDa
Mitochondrial
36kDa
Cytosolic
Lamin A/C Levels
55kDa
Mitochondrial
55kDa
Cytosolic
PLN Levels
25kDa
Mitochondrial
25kDa
Cytosolic
PDI Levels
58kDa
Mitochondrial
58kDa
Cytosolic
Vinculin Levels
116kDa
Mitochondrial
116kDa
Cytosolic
Sham
I/R
I-PostC
P-PostC

Fig.2

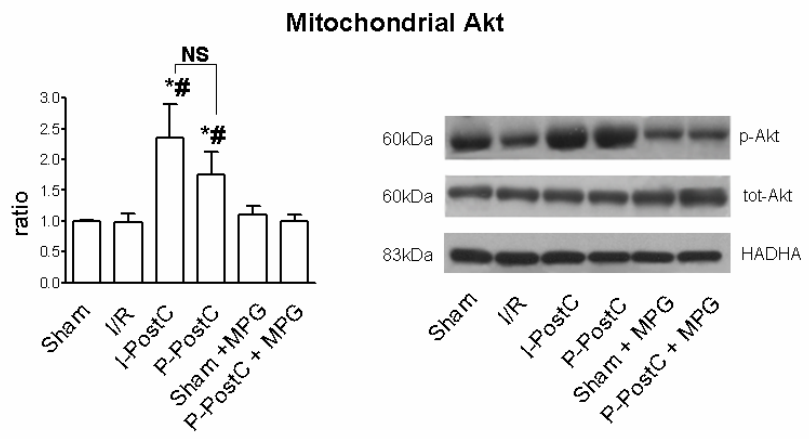
Mitochondrial Akt
NS
*#
*#
ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Sham
I/R
I-PostC
P-PostC
Sham +MPG
P-PostC + MPG
60kDa
p-Akt
60kDa
tot-Akt
83kDa
HADHA
Sham
I/R
I-PostC
P-PostC
Sham + MPG
P-PostC + MPG

Fig.3

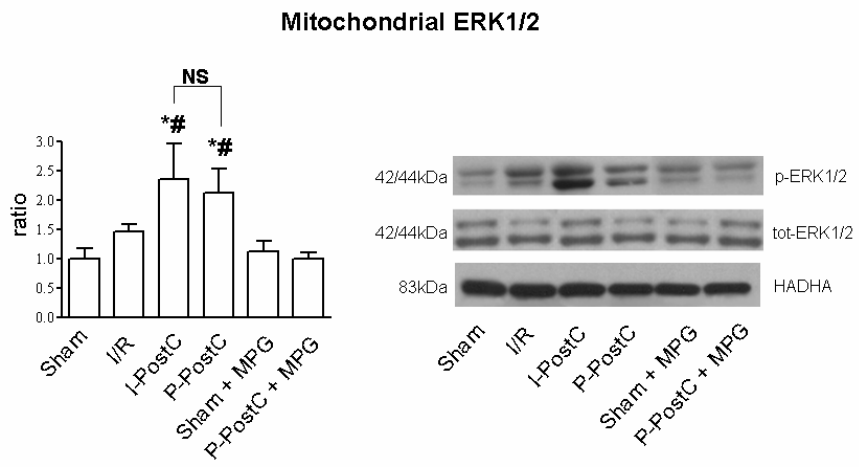
Mitochondrial ERK1/2
NS
*#
*#
ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Sham
I/R
I-PostC
P-PostC
Sham + MPG
P-PostC + MPG
42/44kDa
p-ERK1/2
42/44kDa
tot-ERK1/2
83kDa
HADHA
Sham
I/R
I-PostC
P-PostC
Sham + MPG
P-PostC + MPG

Fig.4

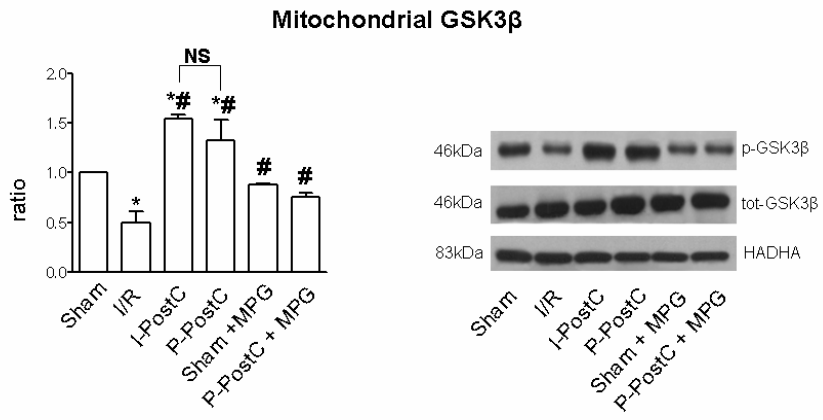
Mitochondrial GSK3β
NS
ratio
2.0
1.5
1.0
0.5
0.0
*
*#
*#
#
#
Sham
I/R
I-PostC
P-PostC
Sham +MPG
P-PostC + MPG
46kDa
p-GSK3β
46kDa
tot-GSK3β
83kDa
HADHA
Sham
I/R
I-PostC
P-PostC
Sham + MPG
P-PostC + MPG

Fig.5

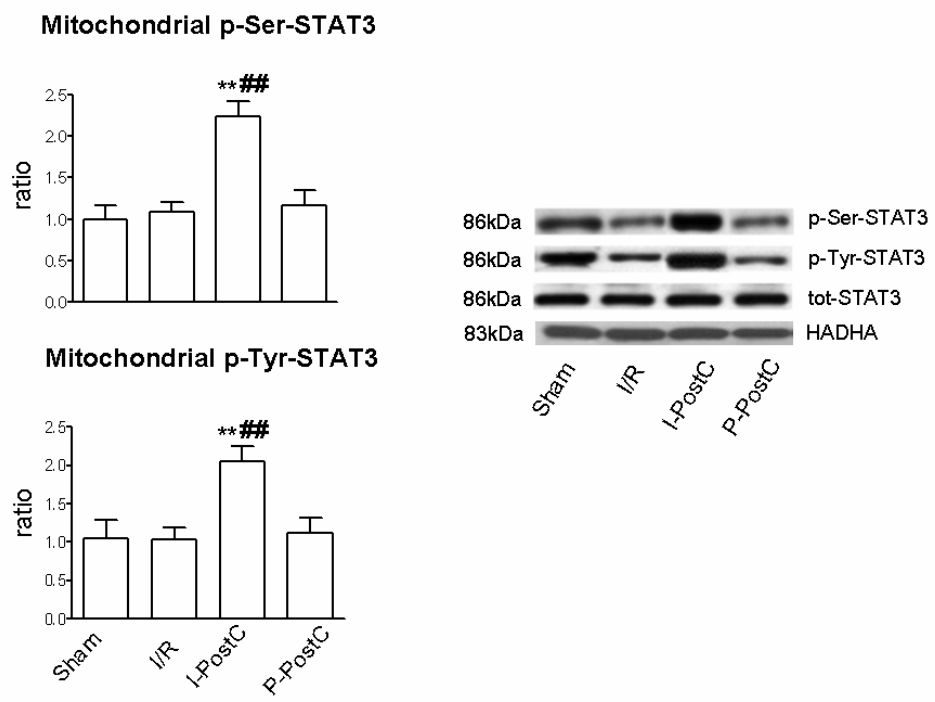
Mitochondrial p-Ser-STAT3
ratio
2.5
2.0
1.5
1.0
0.5
0.0
**##
86kDa
p-Ser-STAT3
86kDa
p-Tyr-STAT3
86kDa
tot-STAT3
83kDa
HADHA
Sham
I/R
I-PostC
P-PostC
Mitochondrial p-Tyr-STAT3
ratio
2.5
2.0
1.5
1.0
0.5
0.0
**##
Sham
I/R
I-PostC
P-PostC

Fig.6

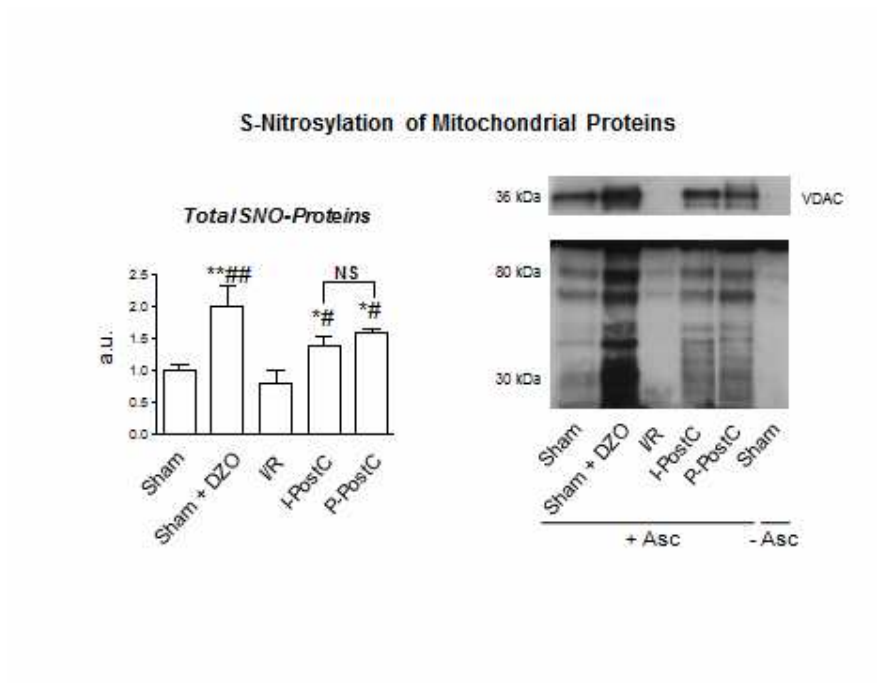
S-Nitrosylation of Mitochondrial Proteins
Total SNO-Proteins
a.u.
**##
NS
*#
*#
Sham
Sham + DZO
I/R
I-PostC
P-PostC
36 kDa
VDAC
80 kDa
30 kDa
Sham
Sham + DZO
I/R
I-PostC
P-PostC
Sham
+ Asc
- Asc

Fig.7

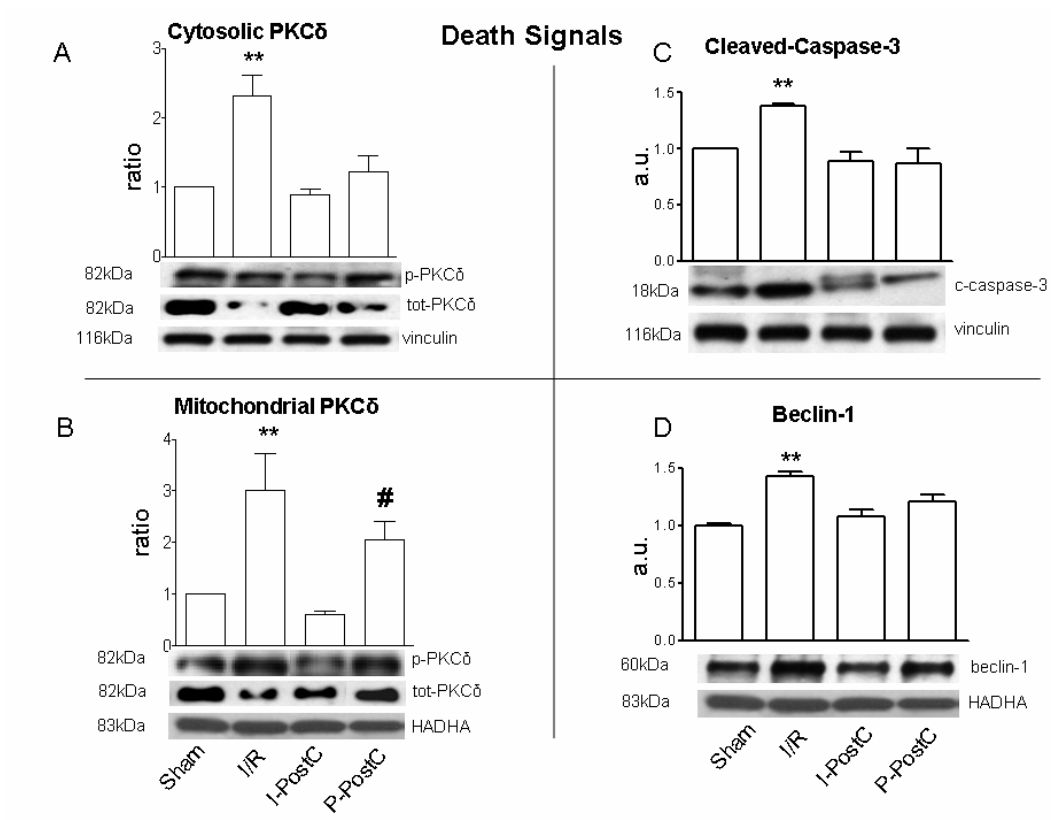
Death Signals
A
Cytosolic PKCδ
ratio
**
82kDa
p-PKCδ
82kDa
tot-PKCδ
116kDa
vinculin
C
Cleaved-Caspase-3
a.u.
**
18kDa
c-caspase-3
116kDa
vinculin
B
Mitochondrial PKCδ
ratio
**
#
82kDa
p-PKCδ
82kDa
tot-PKCδ
83kDa
HADHA
Sham
I/R
I-PostC
P-PostC
D
Beclin-1
a.u.
**
60kDa
beclin-1
83kDa
HADHA
Sham
I/R
I-PostC
P-PostC

Fig.8