Postconditioning induces an anti-apoptotic effect and preserves mitochondrial integrity in isolated rat hearts

Postconditioning Induces an Anti-Apoptotic Effect and Preserves Mitochondrial Integrity in Isolated Rat Hearts

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Abstract

Postconditioning (PostC) may limit mitochondrial damage and apoptotic signaling. We studied markers of apoptosis and mitochondrial protection in isolated rat hearts, which underwent *a*) perfusion without ischemia (Sham), *b*) 30-min ischemia (I) plus 2-hour reperfusion (R), or *c*) PostC protocol (5 intermittent cycles of 10-s reperfusion and 10-s ischemia immediately after the 30-min ischemia). Markers were studied in cytosolic (CF) and/or mitochondrial (MF) fractions. In CF, while pro-apoptotic factors (cytochrome c and caspase-3) were reduced, the anti-apoptotic markers (Bcl-2 and Pim-1) were increased by PostC, compared to the I/R group. Accordingly, phospho-GSK-3β and Bcl-2 levels increased in mitochondria of PostC group. Moreover, I/R reduced the level of mitochondrial structural protein (HSP-60) in MF and increased in CF, thus suggesting mitochondrial damage and HSP-60 release in cytosol, which were prevented by PostC. Electron microscopy confirmed that I/R markedly damaged cristae and mitochondrial membranes; damage was markedly reduced by PostC. Finally, total Connexin-43 (Cx43) levels were reduced in the CF of the I/R group, whereas phospho-Cx43 level resulted in higher levels in the MF of the I/R group than the Sham group. PostC limited the I/R-induced increase of mitochondrial phospho-Cx43. Data suggest that PostC *i*) increases the levels of anti-apoptotic markers, including the cardioprotective kinase Pim-1, ii) decreases the pro-apoptotic markers, e.g. cytochrome c, *iii*) preserves the mitochondrial structure, and *iv)* limits the migration of phospho-Cx43 to mitochondria.

Key words: apoptosis; cardioprotection; ischemia/reperfusion; mitochondria.

Introduction

Cycles of brief reperfusion and ischemia performed immediately at the onset of reperfusion following a prolonged ischemic insult markedly limits reperfusion injury [1-11]. Zhao et al. [1] named this cardioprotective phenomenon "postconditioning" (PostC), which is as powerful as ischemic preconditioning [1-11]. It seems that in rodents PostC protection requires the involvement of several kinases of the reperfusion injury salvage kinase (RISK) pathway, including ERK½, phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB/Akt), protein kinase C (PKC) and glycogen synthase kinase (GSK-3β), as well as nitric oxide synthase (NOS) and guanylyl-cyclase [2-11]. Recently, it has been suggested that PostC attenuates lethal reperfusion injury *via* the opening of mitochondrial K_{ATP} channels (mK_{ATP}) and ROS-signaling, which may lead to a reduction of mitochondrial calcium accumulation, and inhibition of mitochondrial permeability transition pore (mPTP) opening [6,10,11]. Inhibition of mPTP opening [7,10] is reported to be a critical end-effector of the cardioprotective effects of PostC. Therefore mitochondria may play a central role in PostC cardioprotection. Yet, Heusch's group reported that mitochondrial connexin 43 (Cx43) may play a pivotal role in preconditioning, though cardioprotection by postconditioning in Cx43-deficient mice is not lost [12,13]. Nevertheless, Cx43 may play an important role in mitochondrial ROS signaling [12,13].

PostC may limit all forms of cell death (apoptosis, autophagy and necrosis) induced by I/R [14-16]. Apoptosis in reperfusion injury is minor compared to necrosis as indicated by the reduced markers of necrosis (enzyme release and infarct size) in a PostC scenario [1,3,5,6,8,9]. However, apoptosis is an important cause of cell death in the ischemia/reperfusion, *via* both the extrinsic and the intrinsic pathways, which may be accelerated by the bursts of free radicals occurring in reperfusion [14-18]. Therefore reperfusion may represent the principal moment in which apoptosis is present. Since, a cell dying by necrosis may initially show some features of apoptosis, and since both death forms involve mitochondria integrity [14-18], it is important to correlate markers of apoptosis with mitochondria integrity in early reperfusion protection by PostC.

Recently, in a study conducted on *isolated cardiac cells* it has been reported that protection by PostC against apoptosis is mediated by reduced generation of superoxide anions, lowered activity of JNKs/p38, reduced release of TNFα, lowered levels of caspases 8 and 3 and by the modulation of Bax/Bcl-2 ratio [17].

Both in pre- and post-conditioning, PI3K activation is considered an initial step that induces phosphorylation of downstream kinases, which inhibit several pro-apoptotic factors and the mPTP opening at reperfusion [7,18,19]. However, in these protective pathways the anti-apoptotic activity of the cytoplasmic serine/threonine kinase Pim-1 has not been considered, yet. Pim-1 is downstream in the JAK/STAT pathway and plays a crucial role in cardioprotection downstream to Akt [20-23]. Yet, Pim-1 has been reported to have anti-apoptotic activity in myeloid cells and it is considered to be an upstream regulator of bcl-2 expression [21,22]. These data suggest that Pim-1 might also be downstream to PI3K. However, no studies have tested the involvement of Pim-1 in postconditioning. Since the PI3K antagonist(s) may *directly* interfere with Pim-1 activity [20,23], it is necessary to ascertain with a direct method whether or not Pim-1 is involved in PostC. Moreover, markers of mitochondrial integrity (e.g. Heat Shock Protein-60, HSP-60) are not extensively investigated.

Therefore, the principal goal of the present study is to determine whether or not PostC affects markers of mitochondrial integrity and correlated apoptotic factors. In particular, using western blot analysis, we studied, in both cytosol and mitochondria isolated from isolated rat heart, whether or not the PostC protocol affected the levels of pro-apoptotic *cytochrome c* and *caspase-3* as well as protective kinases such as *Bcl-2*, novel *Pim-1* and *phospho-GSK-3β*. Moreover, we studied the level and the status of phosphorylation/dephosphorylation of an important component of the inner mitochondrial membrane (i.e. Cx43) both in mitochondrial and cytosolic fractions. Finally, the preservation of the integrity of mitochondria was evaluated by measuring cytosolic levels of HSP-60, while the ultrastructure of isolated mitochondria was visualized using electron-microscopy imaging.

Materials and Methods

Animals

Male Wistar rats (450-550 g) were treated according to NIH Publication (No. 85-23, revised 1996), and Italian law.

Perfusion technique

Methods used were similar to those previously described [5,6,24]. In brief, isolated rat hearts were retrogradely perfused with oxygenated Krebs-Henseleit buffer at constant flow (9±1 ml/min/g) [5,6,24], 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

After the stabilization period, hearts were randomly divided in three groups: *i)* Sham Group (no-ischemia; n=6), hearts underwent 150-min buffer-perfusion only; *ii)* I/R Group (n=6), hearts underwent 30-min of global-ischemia and then a period of 120-min full-reperfusion; *iii)* PostC Group (n=6), after the 30-min ischemia, hearts underwent a PostC protocol. This consisted of five cycles of 10-s reperfusion and 10-s global ischemia and then heart completed the 120-min full-reperfusion [5,6,24].

Isolation of rat heart mitochondria

Mitochondrial and cytosolic fractions were obtained by a modification of published procedures [10,25-27]. The left ventricle was quickly submerged in 250 mM mannitol, 0.5 mM EGTA, 5 mM Hepes, 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 600g and discarded avoiding solubilization of membrane proteins. Mitochondria were pelleted by 10 min centrifugation at 10300g. The supernatant was centrifuged for 1 h at 100000g to obtain cytosolic fraction. The mitochondrial pellet was suspended, loaded in tubes containing 30% (v/v) Percoll in 225 mM mannitol, 1mM EGTA, 25 mM Hepes, 0.1% (w/v) BSA (pH 7.4) and spun for 30 min at 95000g in a Beckman SW40 rotor. Mitochondria were

collected from the lower part of the dense, brownish yellow mitochondrial band. Protein level in each fraction was determined by Bradford's method [28].

Electron microscopy

To confirm the integrity and purity of mitochondrial fractions, electron microscopy was used [10,27]. Data were also used for a semi-quantitative analysis. The mitochondrial pellet was fixed in 2.5% glutaraldehyde (Fluka, St Louis, MO, USA) and 0.5% saccharose in 0.1 M Sörensen phosphate buffer (pH 7.2) for 2 h. The pellet was then washed in 1.5% saccharose in 0.1 M Sörensen phosphate buffer (pH 7.2) for 6–12 h, post-fixed in 2% osmium tetroxide, dehydrated and embedded in Glauert's embedding mixture, which consists of equal parts of Araldite M and Araldite Härter, HY 964 (Merck), supplemented with 2% of the accelerator DY 064 (Merck). A plasticizer, dibutyl phthalate, was added at 0.5%. Thin sections (70 nm) were cut using a Leica Ultracut UCT, stained with uranyl acetate and lead citrate, and examined in a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for computerized acquisition of images.

Western blotting

About 50µg of protein extracts were separated by SDS-PAGE on 10% (for HSP-60, connexin-43, phospho-connexin-43, GSK-3β, phospho-GSK-3β, caspase-3, Pim-1) or 12% (for cytochrome *c* and Bcl-2) gels and transferred to PVDF membrane (GE-Healthcare). The membranes were incubated overnight with the following primary antibodies: anti-HSP-60 (BD Biosciences), anti-connexin-43 (Sigma), anti-phospho-(Ser-368)-connexin-43, anti-GSK-3β, anti-phospho-(Ser-9)-GSK-3β, anti-caspase-3 (cleaved form), anti-Pim-1, anti-Bcl-2 (Cell Signaling), anti-cytochrome *c* (BD-Pharmingen), all were diluted according to manufacturer instructions. Immunoblotted proteins were visualized by using Immuno-Star HRP Substrate Kit (BioRad) and quantified by Kodak Image Station 440CF. To confirm equal protein loading, blots were

stripped with 0.4M NaOH and then re-blotted with an anti-α-actin antibody (Sigma) for both cytosolic and mitochondrial fraction. Image analyses were performed by the Kodak 1D 3.5 software.

Statistical analysis

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

Results

We studied anti- and pro-apoptosis markers as well as constitutive mitochondrial components in three groups of isolated rat hearts, which underwent *i)* buffer-perfusion without ischemia (Sham), *ii)* 30-min ischemia plus 2-hours reperfusion (I/R), or *iii)* PostC protocol immediately after the 30-min ischemia, i.e. at the onset of reperfusion. Marker/protein levels were studied by Western-blot analysis in cytosolic and/or mitochondrial fractions.

Anti-apoptotic mitochondrial-related factors

As shown in Fig.1A, in cytosolic fraction, the *anti-apoptotic Bcl-2* protein levels were higher in Sham and PostC, than I/R group (26.35±0.13 and 23.95±0.25 *vs* 5.64±0.07 arbitrary units). Also, in mitochondrial fraction, Bcl-2 protein levels were higher in Sham and PostC, than I/R group (15.90±1.80 and 23.23±1.37 *vs* 11.23±2.78 arbitrary units). As shown in Fig.1B, in cytosolic fraction, the level of *protective Pim-1* was also significantly reduced in I/R group with respect to Sham and PostC groups (20.02±0.20 *vs* 35.02±0.13 and 28.84±0.31 arbitrary units). Yet, in mitochondrial fraction *Pim-1* were extremely low in the three experimental conditions. These results suggest that PostC treatments attenuate Bcl-2 and Pim-1 reduction at cytosolic levels, while increasing Bcl-2 mitochondrial levels, thus protecting the heart from I/R induced apoptosis. These results are also in line with those suggesting that Pim-1 acts at cytoplasmatic and nuclear

level both as upstream regulator of bcl-2 expression and as a survival factor to inhibit apoptosis and necrosis [18,22,29].

GSK-3β is a kinase normally expressed in the cytosol of the cells, which is protective when is inhibited by phosphorylation in serine-9. In fact, the *Phospho-GSK-3β*/*total-GSK-3β* ratio increases from 0.56±0.02 in sham to 0.74±0.007 ($p< 0.05$) in PostC groups. Since, it has been suggested that *Phospho-GSK-3β* is translocated to the mitochondria, thus avoiding the mPTP opening that usually occurs in reperfusion [30-32], we checked GSK-3β in both mitochondrial and cytosolic fractions. As shown in Fig. 2A, while in cytosolic fraction, *total GSK-3β* level was higher in the I/R group than the other two groups (10.29±0.80 *vs* 4.58±0.12 and 4.50±0.14 arbitrary units), in the mitochondrial fraction *total GSK-3β* was more expressed in Sham and PostC groups compared to the I/R group (12.02±0.11 and 10.65±0.14 *vs* 6.54±0.81 arbitrary units). As shown, in Fig. 2B, *Phospho-GSK-3β*, in the mitochondrial fraction, was higher in the PostC group with respect to the Sham and I/R groups (5.98±0.10 *vs* 4.72±0.13 and 4.50±0.12 arbitrary units). Yet, in the cytosolic fraction the *Phospho-GSK-3β* level was similar in all three experimental groups.

Data are in agreement with the idea that by enhancing mitochondrial GSK-3β phosphorylation/inactivation in reperfusion phase, PostC may limit mPTP opening and cell death [18,33].

Factors of the mitochondrial apoptotic death pathway

We studied the cytosolic levels of two important factors of the mitochondrial apoptotic death pathway, i.e. the pro-apoptotic *cytochrome c* and *cleaved caspase-3* cytosolic protein levels [14]. As shown in Fig. 3A, in cytosolic fractions *cytochrome c* level was higher in the I/R group (7.16±0.19 arbitrary units) than in Sham and PostC groups (2.96±0.12 and 3.25±0.12 arbitrary units). Data suggest that ischemia/reperfusion promotes the release of cytochrome c from mitochondria to cytosol and that PostC prevents this release. In line with these results, we found that *cleaved caspase-3* cytosolic protein levels (Fig. 3B) were lower in Sham and PostC groups compared to the I/R group (5.24±0.08 and 5.27±0.10 *vs* 18.45±0.16 arbitrary units).

Mitochondrial constitutive proteins

Heat shock protein-60 (HSP-60) and Connexin-43 (Cx43) were studied both in mitochondrial and cytosolic fractions. *HSP-60* is an important constitutive protein of mitochondria [34].

As shown in Fig. 4, in the *mitochondrial fraction* of Sham hearts, HSP-60 level was 33.8±0.58 arbitrary units. HSP level was reduced to 1.6±0.04 in hearts subjected to I/R. The PostC has avoided the reduction in the levels of HSP-60, which were found to be 27.9±0.4 arbitrary units. Data suggest that, in the mitochondria isolated from the I/R group, the level of this protein was almost absent because the integrity of mitochondrial membranes was lost. Yet, the level of HSP-60 in the PostC group was markedly higher than that observed in the I/R group and only slightly lower than that observed in the Sham group; thus suggesting mitochondrial ultra-structure preservation by PostC. In fact, in the analysis of *cytosolic extracts* HSP-60 level was higher in the I/R group with respect to Sham and PostC (13.14±0.79 *vs* 1.25±0.09 and 1.27±0.09 arbitrary units); thus confirming the release of HSP-60 from mitochondria damaged by I/R, but not from mitochondria of PostC group.

Connexin-43 is a member of the family of structurally-related transmembrane proteins, whose phosphorylated portion (Phospho-Cx43) increases with ischemia [35] similarly to p53 [36]. In fact, as shown in Fig 5A, in the mitochondrial fraction *phospho-Cx43* level was higher in the I/R group with respect to Sham group (7.84±0.13 *vs* 0.71±0.19 arbitrary units). PostC treatment limited the increase of phospho-Cx43 to 5.55±0.21 (arbitrary units). In the cytosolic fractions of the three groups there were very low levels of *phospho-Cx43,* which were not influenced by experimental maneuvers.

Yet, as shown in Fig. 5B, *total Cx43* level was higher in the cytosolic fraction of Sham than that of the I/R group (24.00±0.03 *vs* 19.33±0.19 arbitrary units). PostC protocol avoided the reduction in *total Cx43* cytosolic level, which showed similar results to that of the Sham group (28.33±0.19 and 24.00±0.03 arbitrary units). Mitochondrial fraction levels of *total Cx43* were also similar in the Sham and the PostC groups (17.01±0.30 and 19.21±0.20 arbitrary units), while I/R protocol induced an increase of its level (26.12±0.19 arbitrary units).

Data suggests that PostC limits the phosphorylation and translocation of Cx43 to the mitochondria that occurs in I/R. It is likely that ischemia induces an internalization of gap junctional Cx43 and that the cytosolic Cx43 content will be influenced by changes of Cx43 at the gap junctions and of phospho-Cx43 at the mitochondrial level. However, this needs to be ascertained in a future study.

Electron microscopy

As can be seen in panels A and B (Control/Sham), and panels C and D (I/R) of Fig. 6, electron microscopy of isolated mitochondria confirmed that I/R induced marked damage to mitochondrial membranes with cristae disaggregation. In fact the presence of electron dense mitochondrial cristae and well-defined membranes can be observed in about 90% of normal mitochondria, the small percentage of structural disorganization being attributable to the process of mitochondrial extraction. By contrast, I/R caused structural damage in about 80% of mitochondria. Mitochondrial damage can be recognized by the reduced electrondensity of the cristae, presence of broken cristae, and mitochondrial membrane disruption (Panels C, D). The presence of these signs of mitochondrial disorganization was reduced by PostC where the percentage of damaged mitochondria was around 50% (Fig. 6, panels E, F).

Overall data suggest that mitochondrial protection and anti-apoptotic effects are central aspects of PostC protection. Protective kinases (i.e. Pim-1 and phospho-GSK-3β) may play a pivotal role in these protective mechanisms.

Discussion

This study indicates that cardioprotection triggered by PostC depends on the activation/inhibition and/or changes in the level of different kinases related to mitochondria integrity. In fact, PostC enhances phosphorylation/inactivation of mitochondrial GSK-3β. These effects are accompanied by a reduction of cytochrome c release from mitochondria and a reduction of cleaved caspase-3 cytosolic level. Our data also suggest that PostC-induced cardioprotection is characterized by an attenuation of the reduction of the cytosolic levels of Bcl-2 and Pim-1 in the post-ischemic phase. Moreover, PostC mitochondria express more Bcl-2 and less phospho-Cx43 than hearts subjected only to I/R. In the postconditioned hearts mitochondria are less damaged as suggested by the electron microscopy analysis and the preserved level of mitochondrial HSP-60.

Myocardial I/R injury results in robust appearance of cardiomyocyte apoptosis, which contributes to infarct size and long term ventricular dysfunction. The present study considers both in the cytosol and isolated mitochondria fractions of isolated hearts the regulation of several pro- and anti-apoptotic factors together with ultra-structural changes induced by I/R and PostC.

It has been reported in a *cardiomyocyte-like cell line* that PostC reduces apoptosis and that the over-expression of Bcl-2 protects these cells from apoptosis [17,37]. In fact, Bcl-2 proteins attenuate p53-mediated apoptosis in cardiomyocytes [38], increase the Ca^{2+} threshold for mitochondrial transition pore opening, decrease mitochondrial Ca^{2+} efflux due to Na^{+}-dependent Ca^{2+} exchanger in mouse heart mitochondria [39], and inhibit hypoxia-induced apoptosis in isolated adult cardiomyocytes [40]. Importantly, the increase of Bcl-2 may reduce necrotic and oncotic death [18].

Here we confirm, in an isolated rat heart model, that PostC avoids Bcl-2 reduction, which usually accompanies I/R; thus confirming that the anti-apoptotic effect of PostC involves this factor in organ preparation. Our results are in line with the reported anti-apoptotic effect associated with the retention of Ca^{2+} within mitochondria in postconditioned heart [10]; in fact the high level of Bcl-2 may sustain mitochondrial Ca^{2+} retention.

Here we present, for the first time, a role for Pim-1 during PostC protocol. The activation of PI3K/Akt during the PostC protocol has been reported by different groups [2,8,41]. Pim-1 operates downstream of Akt, and a feedback mechanism exists involving the two proteins. Pim-1 inactivation may increase apoptotic activity *via* increased generation of reactive oxygen species and mPTP opening, as found in other cellular contexts [20]. Pim-1 may also act, in part, as a normal upstream regulator of Bcl-2 expression [29]. In a recent study conducted on Pim-1 KO mice, the impossibility to induce cardioprotection by different well known protective agents has been observed [20]. Moreover, cardioprotective agents such as leukemia inhibitory factor, insulin-like growth factor, dexamethasone and phorbol-12-myristate-13-acetate induced Pim-1 immunoreactivity in neonatal rat cardiomyocytes cultures. Importantly, Pim-1 induction by these cardioprotective stimuli was detectable within 2 hours after stimuli application. We demonstrate that PostC avoids the Pim-1 reduction that occurs in I/R. This result is in line with the expression of anti-apoptotic Bcl-2 kinase. These results are also important considering the fact that antagonists of kinases may not be highly specific. In particular, the PI3K antagonist (LY29212) may interfere with Pim-1 activity [20,23]. Therefore, we can not exclude that these inhibitors also interfered with Pim-1, perhaps activated by different pathways (i.e. JAK/STAT) during PostC treatments. In fact this pathway has been proposed as an alternative to the RISK pathway in PostC [42]. Moreover, we can not exclude that protein phosphatase PPA2 may be involved in the change of Pim-1 levels. In fact, PPA2 decreases Pim-1 protein levels [43], enhances I/R injury and limits cardioprotection [44,45]. This hypothesis deserves future studies.

Here we show an enhanced phosphorylation/inactivation of GSK-3β in mitochondria isolated from a PostC group (Fig 2), in agreement with the reported increase in phosphorylation/inactivation of GSK-3β in total samples [46]. This is an important point because GSK-3β phosphorylation represents a pivotal step in the inhibition of mPTP opening during the PostC protocol, as previously reported [7,33,41]. Although the role of GSK-3β phosphorylation in PostC has been recently questioned [47], it is noteworthy that the pro-apoptotic family Bcl-2-Bax is proposed as a novel target of GSK-3β downstream to Akt in different cell lines [18,48].

An important release of cytochrome c occurs at the onset of mitochondrial dysfunction [14]. In fact, in our study the cytochrome c level was elevated only in I/R samples (Fig 2). In the Sham and PostC samples the levels of this marker were lower; thus suggesting that PostC treatments induce early salvage of the mitochondria in reperfusion phase. In fact, antiapoptotic proteins (e.g., Bcl-2, Pim-1) either directly compete with or impede proapoptotic factor activity, stabilizing mitochondrial membranes and their channels thereby preventing mitochondrial disruption [18].

It has been previously shown that the reduction of the level of caspase-3 regulates mitochondrial events both in the initial and the execution phases of apoptosis, such as Bax translocation and cytochrome c release [49]. In the present study cleaved caspase-3 was significantly elevated in the I/R group compared to the Sham and PostC groups; thus suggesting that the PostC induced reduction of I/R injury is in line with a reduced activation of pro-apoptotic kinases.

The analysis of a marker for mitochondrial integrity (i.e. HSP-60) shows that the PostC protocol preserved mitochondria from I/R damage. In fact PostC markedly limited the mitochondrial release of this factor, which, actually, markedly increased in the cytosolic fraction of I/R damaged myocardium. These results are confirmed by the conserved integrity of mitochondrial ultra-structure in PostC hearts, as detected by electron microscopy analysis (Fig. 6).

Cx-43 is predominantly located in the sarcolemma, where six connexins assemble into a so-called connexon or hemichannel [50]. Although migration of Cx43 to mitochondria is controversial, it has also been found in the inner mitochondrial membrane [12,48,51] where it seems to play an important role in reactive oxygen species (ROS) formation [12,13,52]. While Cx43-formed gap junctions are not required for preconditioning protection [50], mitochondrial Cx43 may play an important role in preconditioning induced by ischemia or by diazoxide, which require mitochondrial formation of ROS [13,35,50,51]. Both protections are lost in Cx43 deficient (Cx43+/-) mice. In fact, cardiomyocytes of Cx43+/- mice have a specific functional deficit in ROS formation in response to diazoxide [13,50,51]. Cx43 migrates via TOM/TIM-dependent mechanisms to the mitochondria, where its phosphorylated component increases in the inner

mitochondrial membrane. It is likely that migration and phosphorylation increase during preconditioning treatments and in the reperfusion phase of index ischemia [33,50], when ROS formation occurs. However, PostC protection is not lost in Cx43 deficient (Cx43+/-) mice [12,13,50], thus suggesting that the increase in phospho-Cx43 mitochondrial component is not important in this protection. Here, we confirm that I/R increases the Cx43-phosphorylated mitochondrial component, while reducing the cytosolic component. Yet, we show that PostC limits the reduction of the total cytosolic fraction and limits the increase in the phosphorylated mitochondrial component. It is likely that this limited phosphorylation is not important [26,33,50-52], but may contribute to the limited ROS production, which is, however, necessary for PostC protection [1,6,41,53,54].

Mitochondrial Cx43 exists almost exclusively in the phosphorylated form [35,55,56, present study] indicating that phosphorylation can play an important role in regulating mitochondrial Cx43 content. However, it is not clear whether enhanced expression of phospho-Cx43 in mitochondria by PostC is related to activation of RISKs [26,35,50]. Finally, Heusch' group studied the Cx43 phosphorylation immediately after ischemia with and without ischemic preconditioning [26,51,52], but did not evaluate Cx43 phosphorylation after ischemia and 2 h reperfusion. In this regard, our study and Heusch group's studies are not directly comparable.

The very rapid variation (within minutes) of cytoplasmatic and mitochondrial protein content in response to ischaemia/reperfusion and postconditioning suggests that it is due to either changes in the intracellular distribution, or enzymatic modification (e.g. pro-caspase 3 cleavage) of proteins, rather than to increased protein synthesis. Involvement of mitochondria in PostC is also consistent with the observation that the protective effects of PostC can be mimicked by mK_{ATP} openers and by mPTP blockers [41].

In conclusion, data suggest that mitochondrial protection and anti-apoptotic effects are central aspects of PostC protection. Protective kinases (i.e. Bcl-2, Pim-1 and phospho-GSK-3β) may play a pivotal role in these protective mechanisms. In particular, we report, for the first time, the involvement of Pim-1 in PostC cardioprotection, elucidating a new aspect of signaling with implications for the regulation of cell

survival. The activation of these kinases is accompanied by a reduction of pro-apoptotic elements (cytochrome c and cleaved caspase-3) and preservation/modulation of important components of the mitochondria (HSP-60 and pospho-Cx43). Electron microscopy confirmed that I/R induced marked damages of cristae and mitochondrial membranes, which were largely limited by PostC.

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Figure Legends:

Figure 1. Expression of anti-apoptotic factors in the three experimental groups (Sham, Ischemia/reperfusion (I/R), and Postconditioned groups (PostC); n=6 each). Panel A and Panel B represent, respectively, Bcl-2 and Pim-1 expression in the cytosolic (CF) or mitochondrial fraction (MF). Data are expressed in arbitrary units and normalized for loading control (α-Actin). Panel C shows representative blots.

***** = $p<0.05$ *vs* Sham; **#=** $p<0,05$ vs I/R.

Figure 2. Expression of GSK-3β in the three experimental groups (Sham, Ischemia/reperfusion (I/R), and Postconditioned groups (PostC). Panel A, represents total GSK-3β in the cytosolic fraction (CF) and Panel B, represents the expression of the phosphorylated/inhibited form (Phospho- GSK-3β) in the mitochondrial fraction (MF). Data are expressed in arbitrary units and normalized for loading control (α-Actin). Panel C shows representative blots.

***** = $p<0.05$ *vs* Sham; **#=** $p<0,05$ vs I/R.

Figure 3. Expression of pro-apoptotic factors in the three experimental groups (Sham, Ischemia/reperfusion (I/R), and Postconditioned groups (PostC); n=6 each). Panel A and Panel B represent, respectively, cytochrome *c* and cleaved Caspase-3 in cytosolic fraction (CF). Panel C shows representative blots.

Figure 4. Expression of mitochondrial constitutive protein HSP-60 in the three experimental groups (Sham, Ischemia/reperfusion (I/R), and Postconditioned groups (PostC); n=6 each). HSP-60 data are from cytosolic (CF) and mitochondrial (MF) fractions.

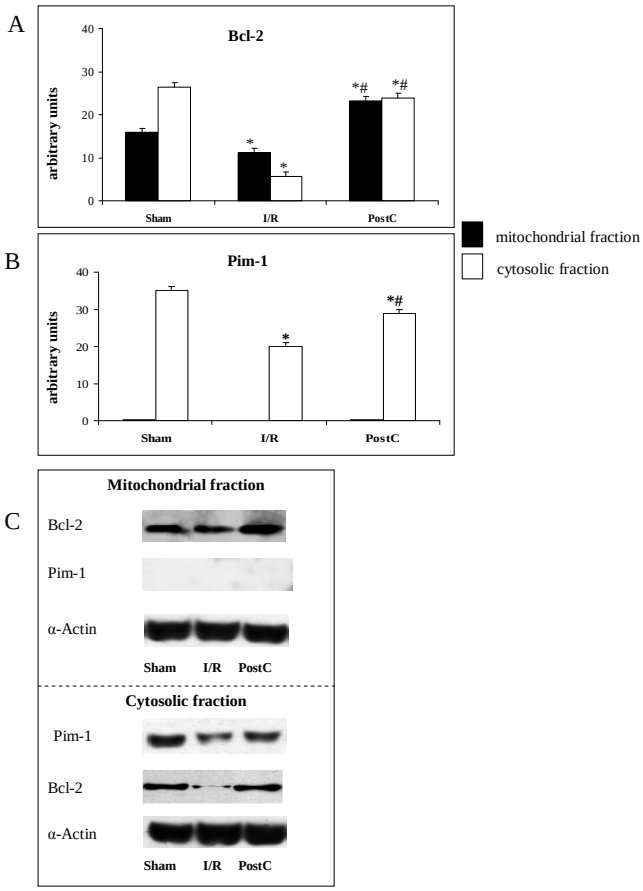
Data are expressed in arbitrary units and normalized for loading control (α-Actin). Panel B shows representative blots.

***** = $p<0.05$ *vs* Sham; **#,** $p<0.05$ *vs* I/R.

Figure 5. Expression of mitochondrial constitutive protein Connexin-43 in the three experimental groups (Sham, Ischemia/reperfusion (I/R), and Postconditioned groups (PostC); n=6 each). Connexin-43 data are from cytosolic (CF) and mitochondrial (MF) fractions. Panel A, phospho-connexin-43; Panel B, total connexin-43. In MF Connexin-43 is detected mainly in the phosphorylated (phospho-connexin-43) form only in heart subjected to I/R with or without PostC. Data are expressed in arbitrary units and normalized for loading control (α-Actin). Panel C shows representative blots.

* = $p<0,05$ *vs* Sham; **#,** $p<0,05$ *vs* I/R.

Figure 6. Electron micrographs of isolated mitochondria from control/sham group (A,B), I/R group (C,D) and PostC group (E,F). Electron microscopy confirmed the integrity and purity of mitochondrial fraction before I/R. While in normal mitochondria (Panels A,B) well-defined membranes and cristae can be observed, as expected, I/R caused mitochondrial damage mainly characterized by extended disruption of mitochondrial membranes and the abundant presence of broken cristae (Panels C,D). The clear reduction in electrondensity of mitochondrial cristae observable in I/R samples is another sign of mitochondrial damage. On the other hand, all ultrastructural signs of mitochondrial damage were drastically reduced by PostC (Panels E,F).


A
Bcl-2
arbitrary units
40
30
20
10
0
*
*
*#
*#
Sham
I/R
PostC
mitochondrial fraction
cytosolic fraction
B
Pim-1
arbitrary units
40
30
20
10
0
*
*#
Sham
I/R
PostC
C
Mitochondrial fraction
Bcl-2
Pim-1
α-Actin
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Cytosolic fraction
Pim-1
Bcl-2
α-Actin
Sham
I/R
PostC

Fig.1

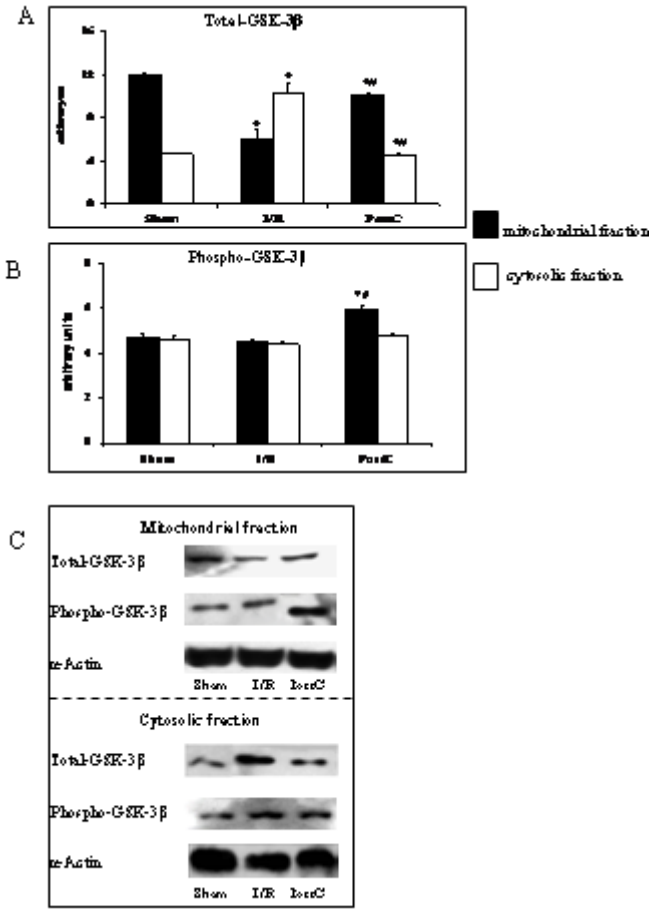
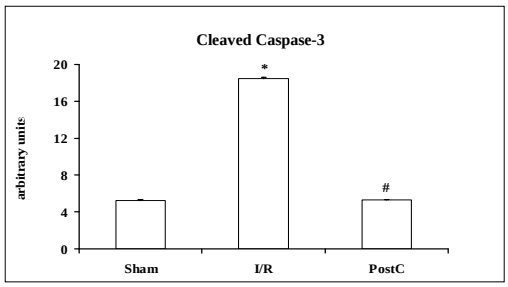
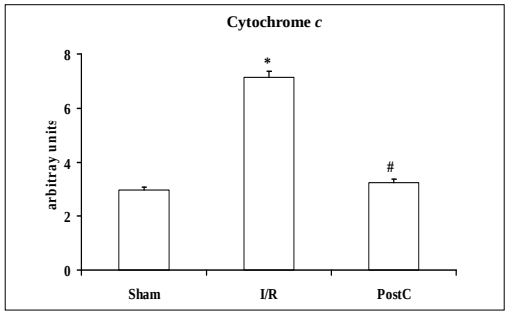

A
Total-GSK-3β
B
Phospho-GSK-3β
mitochondrial fraction
cytosolic fraction
C
Mitochondrial fraction
Total-GSK-3β
Phospho-GSK-3β
α-Actin
Sham
I/R
IorC
Cytosolic fraction
Total-GSK-3β
Phospho-GSK-3β
α-Actin
Sham
I/R
IorC

Fig.2

A


Cleaved Caspase-3
arbitrary units
20
16
12
8
4
0
*
#
Sham
I/R
PostC

B


Cytochrome c
arbitray units
8
6
4
2
0
*
#
Sham
I/R
PostC

C

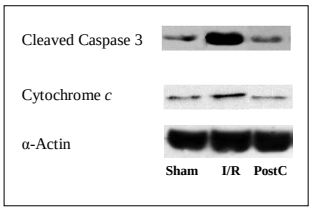
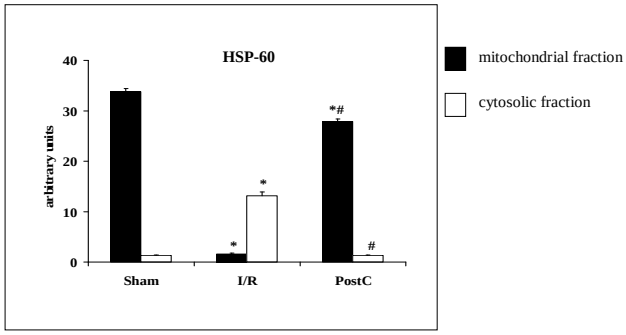

Cleaved Caspase 3
Cytochrome c
α-Actin
Sham
I/R
PostC

Fig.3

A


HSP-60
arbitrary units
40
30
20
10
0
*#
*
*
#
Sham
I/R
PostC
mitochondrial fraction
cytosolic fraction

B

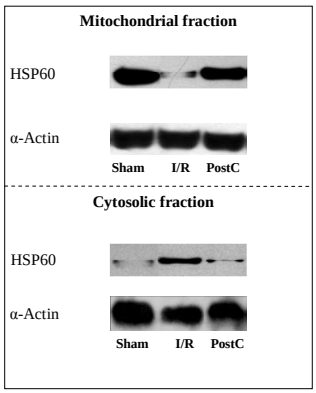

Mitochondrial fraction
HSP60
α-Actin
Sham
I/R
PostC
Cytosolic fraction
HSP60
α-Actin
Sham
I/R
PostC

Fig 4

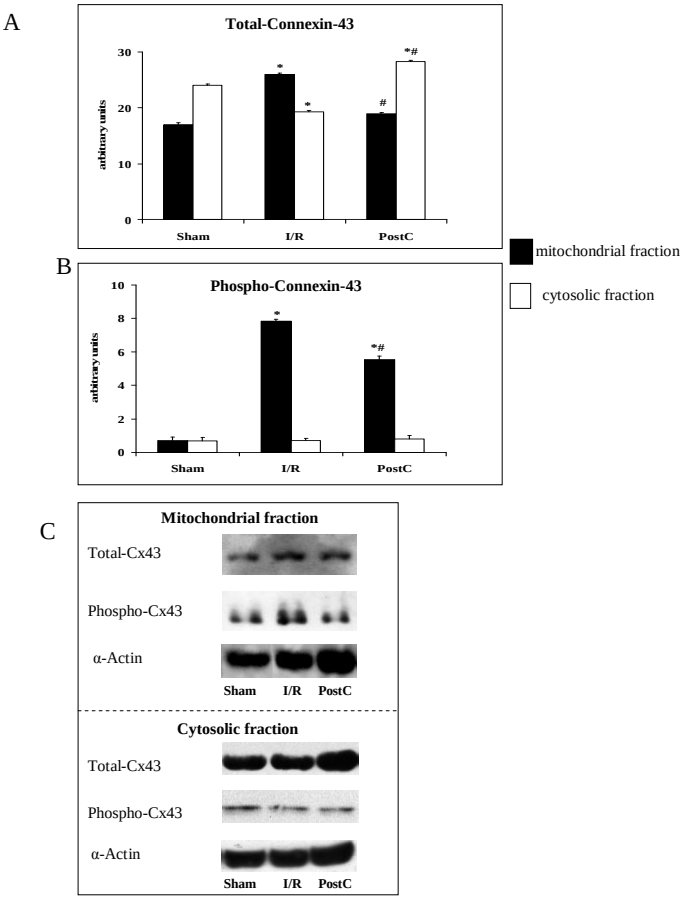

A
Total-Connexin-43
arbitrary units
30
20
10
0
*
*
#
*#
Sham
I/R
PostC
mitochondrial fraction
cytosolic fraction
B
Phospho-Connexin-43
arbitrary units
10
8
6
4
2
0
*
*#
Sham
I/R
PostC
C
Mitochondrial fraction
Total-Cx43
Phospho-Cx43
α-Actin
Sham
I/R
PostC
Cytosolic fraction
Total-Cx43
Phospho-Cx43
α-Actin
Sham
I/R
PostC

Fig.5

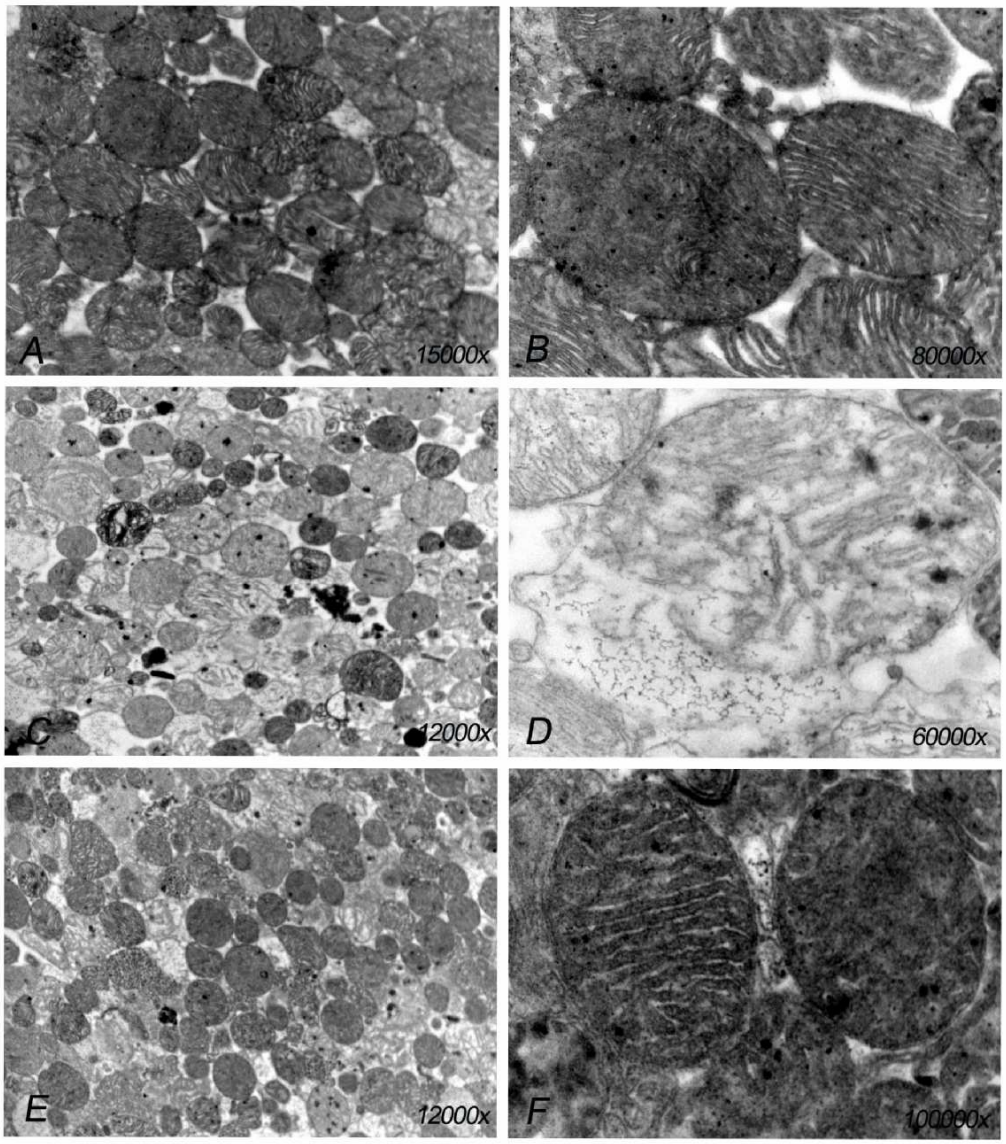


Fig.6