Reperfusion-triggered stress protein response in the myocardium is blocked by post-conditioning. Systems biology pathway analysis highlights the key role of the canonical aryl-hydrocarbon receptor pathway

Gemma Vilahur1†, Judit Cubedo1†, Laura Casani1, Teresa Padro1, Manel Sabate-Tenas4, Juan J. Badimon3, and Lina Badimon1,2*

1Cardiovascular Research Center, CSIC-ICCC, Hospital de la Santa Creu i Sant Pau and CIBEROBN-Pathophysiology of Obesity and Nutrition, c/Sant Antoni MªClaret 167, 08025 Barcelona, Spain; 2Cardiovascular Research Chair, UAB, Barcelona, Spain; 3AtheroThrombosis Research Unit, Mount Sinai School of Medicine, New York, NY, USA; and 4Cath Lab, Div. Cardiology, Hospital Clinic, Barcelona, Spain

Aims
Ischaemic post-conditioning (IPost-Co) exerts cardioprotection by diminishing ischaemia/reperfusion injury. Yet, the mechanisms involved in such protection remain largely unknown. We have investigated the effects of IPost-Co in cardiac cells and in heart performance using molecular, proteomic and functional approaches.

Methods and results
Pigs underwent 1.5 h mid-left anterior descending balloon occlusion and then were sacrificed without reperfusion (ischaemia; n = 7), subjected to 2.5 h of cardiac reperfusion and sacrificed (n = 5); or subjected to IPost-Co before reperfusion and sacrificed 0.5 h (n = 4) and 2.5 h (n = 5) afterwards. A sham-operated group was included (n = 4). Ischaemic and non-ischaemic myocardium was obtained for molecular/histological analysis. Proteomic analysis was performed by two-dimensional electrophoresis followed by matrix-assisted laser desorption/ionization-time-of-flight identification. Potential protein networks involved were identified by bioinformatics and Ingenuity Pathway Analysis (IPA). Cardiac function was assessed by echocardiography. IPost-Co diminished (up to 2.5 h) reperfusion-induced apoptosis of both the intrinsic and extrinsic pathways whereas it did not affect reperfusion-induced Akt/mammalian target of rapamycin (mTOR)/P70 S6K activation. Proteomic studies showed that IPost-Co reverted 43% of cardiac cytoplasmic protein changes observed during ischaemia and ischaemia + reperfusion. Systems biology assessment revealed significant changes in the aryl-hydrocarbon receptor (AhR) pathway (cell damage related). Bioinformatic data were confirmed since the expression of HSP90, AhR, ANRT, and β-tubulin (involved in AhR-signalling transduction) were accordingly modified after IPost-Co. IPost-Co rescued 52% of the left ventricle-at-risk compared with reperfusion alone and resulted in a ≈30% relative improvement in left ventricular ejection fraction (P < 0.05).

Conclusion
IPost-Co improves cardiac function post-myocardial infarction and reduces reperfusion-induced cell damage by down-regulation of the AhR-signalling transduction pathway ultimately leading to infarct size reduction.

Keywords
Ischaemic post-conditioning • Proteomics • Cell damage • AhR pathway

† These authors have contributed equally to this work.
* Corresponding author. Tel: +34 935 565 880, Fax: +34 935 565 559, Email: lbadimon@csic-iccc.org
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Introduction

Reduction of the burden of ischaemia-reperfusion (I/R) injury is a major challenge of several treatments for cardiovascular diseases. Most of the detrimental effects of I/R are triggered within the first few minutes following the reopening of the occluded artery and overall lead to irreversible cardiomyocyte death. Yet, apoptosis is also executed upon reperfusion likely contributing to the final extent of myocardial infarction. Unlike many interventions that have proven to afford cardiac protection in animal models and have failed to do so in humans, the phenomenon of ischaemic pre-conditioning (brief periods of I/R before sustained ischaemia) has been shown to protect the human myocardium in several clinical presentations of I/R including cardiac surgery, pre-infarction angina, and angioplasty. However, the clinical utility of ischaemic pre-conditioning is limited by the need to apply such intervention prior to acute infarction. Interestingly, within the last decade, many experimental and proof-of-concept human studies have supported that the application of a similar regimen immediately after sustained ischaemia (ischaemic post-conditioning, IPost-Co) exerts cardioprotective effects limiting infarct size. Ischaemic post-conditioning-related cardioprotective mechanisms are currently under investigation. Emerging data are providing evidence that IPost-Co-triggered cytoplasmic effects mostly converge to inhibit the mitochondrial permeability transition pore (MPTP) in order to partly rescue jeopardized cardiomyocytes. For instance, IPost-Co has shown to activate via membrane-receptor the pro-survival signal transduction reperfusion injury salvage kinase (RISK)-pathway-preventing MPTP opening thus limiting irreversible cardiac injury. Nevertheless, whereas most studies in rodents and isolated ischaemic hearts have clearly demonstrated that infarct size reduction by IPost-Co is RISK dependent, recent studies in pigs have failed to implicate components of the RISK pathway as mediators of IPost-Co protection and/or protocols used (ischaemia severity and duration, IPost-Co algorithms, etc.). More importantly, such observations support the need for further studies in clinically relevant experimental approaches/models to unravel the alterations and signal transduction pathways that may account for the detected cardioprotection afforded by IPost-Co in the clinical scenario. As such, a recent study in rats has shown the capability of IPost-Co to reduce the rate of myocyte apoptosis likely via RISK-ImTOR-dependent mechanisms. However, whether IPost-Co diminishes apoptosis in a human-resembling experimental model remains to be addressed. In this regard, we have recently reported in pigs that reperfusion is the main trigger of caspase-3 in the setting of I/R. On the contrary, although IPost-Co has already shown to reduce infarct size, the effect of IPost-Co on cardiac performance and on the mechanisms underlying its protective effects remains largely unknown.

We sought to evaluate, in a pig model of I/R, the effect of IPost-Co in cardiac cell damage and on cardiac performance. To this end we have evaluated, by proteomics, the coordinated changes that occur in myocardial-related cytoplasmic proteins upon IPost-Co and by echocardiographic assessment cardiac performance.

Methods

For expanded and detailed materials and methods please refer to the Supplementary material online, file.

Experimental model

Twenty-five swines were acclimated for 1 week before any experimental procedure. Thereafter, 21 animals were randomized to one of the following four groups: (I) closed-chest 1.5 h left anterior descending (LAD) coronary occlusion with no reperfusion (n = 7); (II) 1.5 h LAD occlusion followed by 2.5 h reperfusion (n = 5); (III) 1.5 h LAD occlusion followed by IPost-Co and 0.5 h reperfusion (n = 4), and (IV) 1.5 h LAD occlusion followed by IPost-Co and 2.5 h reperfusion (n = 5). The IPost-Co protocol was induced by six cycles of 20 s of reperfusion and 20 s of re-occlusion at the onset of reperfusion as previously reported. A sham-operated group of animals (n = 4) following the same operating procedure without ischaemia was included.

The study protocol was approved by an institutional animal research committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Myocardial ischaemia/reperfusion model and echocardiography

Acute myocardial infarction (AMI) was experimentally induced as we have previously described. Briefly, anaesthesia was induced by intramuscular injection of zoletil® (7 mg/kg), domitor® (7 mg/kg), and atropine (0.03 mg/kg). Animals underwent endotracheal intubation, and anaesthesia was maintained by isofluorane inhalation (2%). Continuous infusion of amiodarone (300 mg, 75 mg/h) was initiated at the beginning of the procedure in all pigs as prophylaxis for malignant ventricular arrhythmias. These amiodarone doses do not alter haemodynamic parameters. Angiographic images were used to guide angioplasty balloon placement (below the first diagonal branch) and balloon occlusion was maintained for 1.5 h. Thereafter, animals were distributed to one of the four groups described above. A sham-operated group was also performed.

Heart rate and electrocardiogram were monitored throughout the experimental procedure. We used two-dimensional echocardiograms (2DE) (Phillips iE33) to assess left ventricular ejection fraction (LVEF) in all animals before coronary occlusion (baseline), 1.5 h post-ischaemia (before reperfusion), and at the end of the reperfusion period (sacrifice). In order to reduce the variability echocardiographic examinations were all performed by the same professional trained in echocardiographic measurements and blind to experimental approaches.

Sample collection

Evan’s Blue dye was injected in anaesthetized animals to outline the area-at-risk (AAR) after which the animal hearts were arrested, rapidly excised. Hearts were sectioned so that consecutive slices were alternatively collected for infarct size analysis (triphenyl tetrazonium chloride, TTC; see below) and cellular/molecular/proteomic studies of the ischaemic (IM) and non-ischaemic (NIM) myocardium.
Morphometric determination of infarct size by triphenyl tetrazolium chloride

The impact of IPost-Co on limiting infarct size was evaluated by TTC staining that was performed in the excised myocardium of those animals allowed to be reperfused for 2.5 h (with and without IPost-Co) to ensure an accurate infarct size assessment.24

Protein extraction from myocardial tissue

Frozen myocardial tissue samples from the IM and NIM of all animal groups were pulverized and homogenously distributed in different aliquots for RNA (Trizol© isolation reagent) and protein extraction (lysis buffer). For proteomic studies, pulverized tissue from the IM was homogenized in urea/thiourea buffer and protein concentration was measured with 2D-Quant Kit.

Mitochondrial extracts were obtained as previously described for apoptotic marker analysis.25

Molecular analysis

Gene expression analysis

We analysed by real-time polymerase chain reaction (PCR; Applied Biosystems and TaqMan RT-PCR) mRNA levels for: (i) apoptotic-related markers of both the extrinsic [Fas Receptor/CD95, caspase-8, and nuclear factor kappa B (NFκB)] and intrinsic (Bax, Bcl-2, and P53) pathways and the final irreversible executor caspase-3; (ii) the RISK component phosphoinositide 3-kinase (PI3K); and (iii) the aryl-hydrocarbon nuclear translocator (ARNT or Hif1β).

Western blot analysis

The IM and NIM extracts were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted to nitrocellulose membranes and incubated with: (i) apoptosis markers (truncated-caspase-3 and p53 phosphorylated on Thr155); (ii) RISK pathway (Akt/ protein kinase B (PKB) phosphorylated on Ser473); Akt/PKB); (iii) myocyte hyperthrophy pathway (mTOR phosphorylated at Ser2448 and P70S6K phosphorylated at Thr389); and (iv) aryl-hydrocarbon nuclear receptor (AhR). β-Actin was used for protein loading control. Isolated mitochondrial extracts were processed similarly and membranes incubated against Bcl-2 phosphorylated at Ser87.

Caspase-8 activity assessment

Caspase-8 activity was also assessed in the IM following the manufacturer's instructions, read by optical density at 405 nm, and expressed as arbitrary units.

2′-deoxyuridine, 5′-triphosphate nick-end labelling staining

Apoptosis assessment in the IM was also performed by 2′-deoxyuridine, 5′-triphosphate (dUTP) nick-end labelling (TUNEL) using an apoptosis detection kit according to the manufacturer's protocol (Chemicon Inc.). The number of TUNEL-positive cardiomyocytes was expressed as mean number/100 cells/microscopic field (mean 5 fields/animal). Around 140 cells were counted per field.

Proteomic analysis: two-dimensional gel electrophoresis and mass spectrometry analysis

Myocardial proteomic changes induced by ischaemia and full reperfusion (2.5 h) with respect to sham-operated animals were firstly investigated and then compared with that of IPost-Co. To that end, protein extracts from the IM of sham-operated animals, animals subjected to ischaemia, ischaemia + 2.5 h reperfusion and ischaemia + IPost-Co + 2.5 h reperfusion were separated by 2DE. Three animals from each group were analysed. Spot patterns were analysed for differences using PD-Quest 8.0 (BioRad). Protein spots of interest were excised for identification by matrix-assisted laser desorption/ionization-time-of-flight.24,26

In silico bioinformatic analysis

A systems biology approach was applied with Ingenuity System Pathway Analysis (IPA) software to determine the statistically significant neural networks and canonical pathways in which the identified proteins were involved.

Statistical analysis

Because data were not normally distributed as observed by applying the Shapiro-Wilk test, a non-parametric statistical analysis was applied and results are reported as medians and interquartile range [IQR]. For independent factors (comparisons between groups) we performed Kruskal–Wallis and Mann–Whitney analysis; for repeated measurements (ischaemia vs. non-ischaemia and different time-points) Wilcoxon and Friedman analysis. All statistical tests conducted were two sided and P < 0.05 was considered significant. Statistical analysis was performed using SPSS (version 19.0).

Results

Ischaemic post-conditioning limits infarct size

The AAR was similar among the different animal groups achieving 34% [32–35] of the LV.

Infarct size was 87.9% [86.8–90.6] of the AAR in 2.5 h reperfused animals and 36.2% [32.3–39.5] of the AAR in IPost-Co + 2.5 h reperfusion animals. IPost-Co, then, resulted in a 52% salvage of the left ventricle-at-risk as compared with reperfusion alone (P < 0.05).

Beneficial effect of ischaemic post-conditioning on cardiac contractile function

All groups showed a similar mean heart rate throughout the procedure (Table 1A). As to cardiac performance, 1.5 h of ischaemia markedly and similarly diminished LVEF in all animals visualized by a marked akinesia. No changes in global cardiac function were detected in those subjected to reperfusion (Table 1B). In contrast, IPost-Co resulted in a 10% cardiac improvement (at 0.5 h-reperfusion P = 0.067; at 2.5 h-reperfusion P < 0.05), which corresponds to an almost 30% relative increase in LVEF, respectively, with respect to all the other animals.

Ischaemic post-conditioning halts reperfusion-induced apoptosis execution

Real-time polymerase chain reaction analysis

Gene expression levels in the NIM remained unaltered among the different animal groups. In the IM, IPost-Co prevented reperfusion-induced up-regulation of several genes encoding for molecules downstream to the death receptor pathway and to the mitochondrial-related apoptosis pathway (Figure 1A and B, Table 1C).
Table 1  Median [IQR] of heart rate and cardiac global function (assessed by left ventricular ejection fraction) of all animals at the end of the different tested time-points

| A: Heart rate | Baseline (pre-I/R) | Sacrifice |
|---------------|--------------------|-----------|
| I             | 75 [74–76]         | 78 [72–80]|
| 1 + 2.5 h R   | 73 [71–75]         | 70 [69–72]|
| I + IP + Co + 0.5 h R | 75 [73–77] | 73 [72–74] |
| I + IP + Co + 2.5 h R | 76 [74–77] | 70 [69–72] |

| B: Cardiac global function | Baseline (pre-I/R) | 1.5 h post-AMI | Sacrifice | % Reduction vs. baseline | % Reduction vs. post-AMI |
|----------------------------|--------------------|----------------|------------|--------------------------|--------------------------|
| I                          | 77 [71–80]         | 40 [39–47]*   | 40 [39–47]*| 30 [29–35]               | 0 [0–0]                  |
| 1 + 2.5 h R                | 71 [69–72]         | 36 [33–38]*   | 36 [33–38]*| 33 [32–36]               | 0 [0–0]                  |
| I + IP + Co + 0.5 h R      | 71 [67–74]         | 35 [34–36]*   | 46 [43–49]*| 35 [32–37]               | −12 [−13 to −9]          |
| I + IP + Co + 2.5 h R      | 66 [64–70]         | 35 [35–35]*   | 44 [44–45]*| 31 [27–35]               | −9 [−10 to −9]           |

*P < 0.05 vs. baseline; **P < 0.05 vs. 1.5 h post-AMI. AMI, acute myocardial infarction; I, ischaemia; R, reperfusion; IP + Co, ischaemic post-conditioning.

Figure 1  Effect of IP + Co on gene expression of different components of the extrinsic (A) and intrinsic (B) apoptotic pathway and caspase-3 (C) within the IM (grey boxes) and NIM (white boxes) myocardium. *P < 0.05 vs. the IM all other groups; †P < 0.05 vs. NIM. Data are represented as box plot diagrams [1.5 h I, n = 7; 1.5 h I + 2.5 h R, n = 5; 1.5 h I + Post-Co + 0.5 h R, n = 4; 1.5 h I + Post-Co + 2.5 h R, n = 5]. I, ischaemia; R, reperfusion; IP + Co, ischaemic post-conditioning; IM, ischaemic; NIM, non-ischaemic.
respectively) preventing caspase-3 increase (Figure 1C). Indeed, Fas-receptor/CD95, caspase-8, NFκB, Bax, Bcl-2, and P53 expression levels remained unchanged in all IPost-Co animals with respect to animals subjected to ischaemia alone and similar to that of NIM tissue (Figure 1).

**Caspace-8 activity assessment**

Ischaemic post-conditioning prevented the increase in caspase-8 activity within the IM detected in reperfused animals (0.31 [0.29–0.35] AU) leaving, at 2.5 h of post-reperfusion levels comparable to that of animals subjected to ischaemia alone (0.10 [0.08–0.13] vs. 0.10 [0.07–0.11] AU).

**Protein analysis by western blot**

The limited availability of anti-porcine-protein antibodies does not permit analysis of the protein expression pattern of all the evaluated genes. Yet, we could observe that mitochondrial expression of P-Bcl-2, protein levels of active P53 and activation of caspase-3 (Figure 2A) that were markedly enhanced in IM by reperfusion (P < 0.05), remained unchanged in all those animals subjected to IPost-Co (P = 0.754 vs. ischaemia alone) and with expression levels similar to those encountered in the NIM.

### Table 2  Fold change of spot intensity in two-dimensional electrophoresis analysis of sham, ischaemia/reperfusion, and ischaemic post-conditioning animals

| Protname                  | I/S | I + R/I | I + R/S | IPost-Co/I + R | IPost-Co/S |
|---------------------------|-----|---------|---------|----------------|------------|
| Alpha actin               | ↑1.9| 1.0     | ↑1.8    | ↑1.6           | ↑2.8       |
| Annexin A1                | ↓1.1| ↓1.8    | ↓2.0    | ND IPost-Co    | ND IPost-Co|
| Annexin A11               | ↓1.2| ↓1.8    | ↓2.0    | ↑5.3           | ↑2.5       |
| Annexin A2                | ↑1.5| ↓1.8    | ↓1.3    | ↑3.4           | ↑2.8       |
| Cardiac phospholamban     | ↑1.2| ↓1.5    | ↓1.3    | ↑2.6           | ↑2.1       |
| Cardiac troponin T isoform 1 | ↓5.8| ↑2.1    | ↓2.5    | ↑2.6           | ↓1.1       |
| Creatine kinase B-type    | ↓2.8| ↑1.4    | ↓2.0    | ND IPost-Co    | ND IPost-Co|
| Creatine kinase M-type    | ↓1.7| ↓1.3    | ↓2.0    | ↑14.2          | ↑6.4       |
| Creatine kinase S-type    | ↓2.4| ↓1.1    | ↓2.5    | ↑5.1           | ↑1.9       |
| Cytosolic aspartate aminotransferase | 1.0 | ↓1.6    | ↓1.7    | ↑2.0           | ↑1.3       |
| Glutathione S-transferase | ↓1.3| ↓1.1    | ↓1.4    | ↑1.9           | ↑1.4       |
| Lactate dehydrogenase     | ↓1.4| ↑1.3    | ↓1.1    | ↑1.4           | ↑1.2       |
| Malate dehydrogenase      | ↓1.1| ↓1.8    | ↓2.0    | ↑2.7           | ↑1.4       |
| Myosin light chain 2v     | ↓1.7| ↓1.1    | ↓1.7    | ↑4.4           | ↑2.5       |
| Myosin light chain 3      | ↑1.3| ↓2.0    | ↓1.4    | ↑3.0           | ↑2.0       |
| PTEN                      | 1.0 | 1.0     | ↑1.1    | ND IPost-Co    | ND IPost-Co|
| Rab GDP dissociation inhibitor beta | ↓1.4| ↓1.2    | ↓1.7    | ↑3.4           | ↑1.4       |
| Spectrin beta chain, brain 4 | 1.0 | ↑1.4    | ↑1.3    | ↑1.6           | ↑2.1       |
| Tubulin beta chain        | ↓2.5| ↓1.1    | ↓3.3    | ↑3.1           | 1.0        |
| T-complex protein subunit epsilon | ↓1.3| ↓1.6    | ↓2.0    | ↑2.9           | ↑1.4       |
| Hsp70 (kDa)               | ↓1.2| ↓2.0    | ↓2.5    | ↑3.7           | ↑1.6       |
| Hsp90 (kDa)               | ↓2.4| ↑1.8    | ↓1.4    | ↓3.6           | ↓5.0       |
| Hsp-β2                    | ↓1.3| ↓1.2    | ↓1.4    | ↑2.5           | ↑1.7       |
| Endoplasmic               | ↓1.3| ↑1.4    | ↑1.1    | ↓2.0           | ↓1.9       |

I, ischaemia; R, reperfusion; IPost-Co, ischaemic post-conditioning; ND, not detected; Hsp, heat-shock protein; S, Sham; PTEN, phosphatase and tensin homologue.

**2′-deoxyuridine, 5′-triphosphate nick-end labelling staining**

As shown in Figure 2B and in agreement with the detected levels of the apoptotic executor truncated caspase-3, IPost-Co resulted in low apoptotic cell counts within the IM identified by TUNEL staining as compared with I/R. The group of animals only subjected to ischaemia, as expected, also displayed a low TUNEL detection.

**Ischaemic post-conditioning effects on mammalian target of rapamycin pathway activation**

Ischaemic post-conditioning animals at 0.5 h post-reperfusion showed an increase in PI3K and in Akt/mTOR/P70S6K phosphorylation that was comparable to that of animals subjected to 2.5 h reperfusion without IPost-Co (Figure 3A and B). However, IPost-Co animals at 2.5 h post-reperfusion showed reduced levels of protein phosphorylation. No changes were observed in NIM.

**Effect of ischaemic post-conditioning in swine myocardial proteome**

Proteomic analysis of IM revealed that at least 23 cytoplasmic proteins were differentially expressed among the different groups...
Figure 2  Protein analysis and representative images of mitochondrial P-Bcl-2, P-p53 and truncated caspase-3/total caspase-3 (A) Apoptosis analysis in the ischaemic cardiac region by 2'-deoxyuridine, 5’-triphosphate nick-end labelling staining (apoptosis: red) and representative images; I: 1.5 h I; II: 1.5 h I + 2.5 h R; III: 1.5 h I + Post-Co + 0.5 h R; IV: 1.5 h I + Post-Co + 2.5 h R. (B) *P < 0.05 vs. the IM all other groups. †P < 0.05 vs. NIM. Data are represented as box plot diagram (1.5 h I, n = 7; 1.5 h I + 2.5 h R, n = 5; 1.5 h I + Post-Co + 0.5 h R, n = 4; 1.5 h I + Post-Co + 2.5 h R, n = 5). I, ischaemia; R, reperfusion; IPost-Co, ischaemic post-conditioning; IM, ischaemic; NIM, non-ischaemic.
Spot intensity values were normalized by the intensity of albumin spot in each gel. The spot intensity in each group was calculated as the median [IQR] of the intensity obtained in the analysis of each independent animal (Supplementary material online, Table S2). Ischaemia induced the decrease in the intensity of 7 proteins being α-actin

**Figure 3** Effect of IPost-Co on phosphoinositide 3-kinase gene expression (A) and activation of the Akt/mTOR/P70S6K pathway (B) in the IM (grey boxes) and NIM (white boxes) myocardium. *P < 0.05 vs. the IM of 1.5 h I and 1.5 h I + Post-Co + 2.5 h. †P < 0.05 vs. NIM. Data are represented as box plot diagram (1.5 h I, n = 7; 1.5 h I + 2.5 h R, n = 5; 1.5 h I + Post-Co + 0.5 h R, n = 4; 1.5 h I + Post-Co + 2.5 h R, n = 5). I, ischaemia; R, reperfusion; IPost-Co, ischaemic post-conditioning; IM, ischaemic; NIM, non-ischaemic.
the only protein showing more than 1.7-fold increase in its intensity. Ischaemia/reperfusion was associated with a reduction (1.7- to 3.3-fold) in the intensity of 13 cytoplasmic proteins to the levels below sham-operated animals. A strong effect was detected in β-tubulin (3.3-fold decrease). α-Actin was still 1.8-fold above sham-operated animal levels after reperfusion.

Ischaemic post-conditioning induced an up-regulation of cytoplasmic proteins. As such, the intensity of 10 proteins was more than 1.7-fold increased when compared with the sham-operated group, being 8 of these proteins also increased over 1.7-fold when compared with I/R group. The strongest change induced by IPost-Co referred to the CK-M type (Q5XLD3) that was 14- and 6-fold increased when compared with the I/R and the sham-operated groups, respectively. In addition, two stress-related cytoplasmic proteins were markedly down-regulated by IPost-Co. Those proteins were Hsp90 and endoplasmic with a 5- and a 19-fold decrease, respectively, as compared with the sham-operated group. After IPost-Co, 6 cytoplasmic proteins that were down-regulated in I/R were normalized reaching sham levels. β-Tubulin levels were normalized after IPost-Co.

**Figure 4** Ingenuity Pathway Analysis network showing the relation between the identified cytoplasmic proteins in ischaemic myocardial tissue. Red-depict proteins up-regulated and green-depict proteins down-regulated due to ischaemia. Protein networks are significantly associated with apoptosis (P < 0.0001).

**In silico bioinformatic analysis: involvement of aryl-hydrocarbon receptor pathway**

Bioinformatic analysis using the IPA software depicted that proteins showing changes due to ischaemia were significantly involved in the molecular and cellular function pathway related to apoptosis (P < 0.05; Figure 4). Identified proteins were significantly associated to the canonical pathway involved in AhR signalling (P < 0.0001; Figure 5A), with significant changes detected in the different groups of animals. Ischaemia induced a down-regulation of Hsp90, an up-stream effector of the AhR pathway (Figure 5B) while reperfusion induced an up-regulation (Figure 5C).

When IPost-Co was compared with ischaemia (Figure 5D) and I/R (Figure 5E) a strong down-regulation of Hsp90 was evident. If IPost-Co was compared with sham-operated animals, Hsp90 was still under normal levels (Supplementary material online, Figure S1A). Reperfusion induced up- and down-regulation of different subunits of Hsp90 (Supplementary material online, Figure S1B) when compared with sham-operated animals.
Aryl-hydrocarbon receptor and aryl-hydrocarbon nuclear translocator changes in cardiac tissue

To experimentally validate the in silico findings on AhR involvement in IPost-Co, myocardial tissue extracts were specifically analysed for AhR changes by western blot (Figure 5F). The I/R induced a 3-fold increase in AhR when compared with ischaemia alone, and 6-fold increase when compared with sham-operated animals ($P < 0.05$). The strongest change was seen in IPost-Co where AhR band intensity was 21- and 16-fold reduced (for IPost-Co + 0.5 h R and IPost-Co + 2.5 h R, respectively; $P < 0.05$) in comparison with I/R animals confirming the results obtained in the in silico analysis. To further assess the role of the AhR signalling pathway, RNA expression of the ARNT was analysed (Figure 5G). IPost-Co prevented reperfusion-induced ARNT increase. Specifically, reperfusion was associated with a 4.4-fold change vs. sham and 3.4-fold change vs. ischaemia ($P < 0.05$ for both).

Discussion

In a pig model of 1.5 h coronary ischaemia and 2.5 h reperfusion, our data show that in contrast to fast-onset reperfusion, IPost-Co prevents cardiac extrinsic (Fas-induced) and intrinsic (mitochondrial) apoptotic pathway activation. Moreover, by proteomic approaches and in silico bioinformatic analysis we have shown for the first time that the cytoplasmic-related Hsp90/AhR signalling transduction pathway seems to be involved in the detected IPost-Co protective effects. The clinical evaluation of the hearts showed that the IPost-Co is associated with an acute amelioration in global cardiac performance ultimately limiting the infarct size.

IPost-Co has shown to exert cardioprotection and reduce infarct size in both experimental and clinical studies. In fact, IPost-Co is the first method proven to reduce the final infarct size by $\sim50\%$ in several in vivo models. The benefit of IPost-Co on cardiac function/recovery in the setting of I/R has not been extensively studied. Studies in cats and rodents have reported...
the capability of IPost-Co to attenuate reperfusion-related ventricular arrhythmias following a short ischaemic episode.\textsuperscript{28,29}

We provide further evidence, by ultrasound assessment, that IPost-Co after severe ischaemia exerts a beneficial effect on global cardiac performance indicating the potential of IPost-Co to attenuate post-ischaemic myocardial stunning and favour the recovery of the left ventricle contractile performance.

The mechanisms and signal transduction pathways behind such cardioprotective effects remain largely unknown. Rodent studies have suggested the involvement of RISK and mTOR activation in the salvage of jeopardized myocardium. However, recent previously published data by Heush et al.\textsuperscript{18} indicates that there seems to be no causal role for RISK and/or Akt/mTOR/P70S6K pathways activation in the cardioprotection afforded by IPost-Co in swine, an animal model of higher human resemblance than rodents. The authors reported that RISK acutely increases within the first 30 min and then declines to baseline levels, as we also find in our studies. Interestingly, they provide evidence that RISK blockade (with Wortmannin and U0126) does not affect IPost-Co-derived cardioprotection. Thereby, these results seem to indicate that RISK is not critical in IPost-Co. In fact, Heusch et al. have recently reported the involvement of STAT3 in mediating IPost-Co cardio-protection by preserving mitochondrial function.\textsuperscript{20} We suggest that IPost-Co may also limit lethal reperfusion injury by preventing apoptosis execution. Indeed, I/R is a complex phenomenon that mostly converge in mitochondrial modulation of cell death. Our proteomic studies revealed important changes in the proteomic profile during ischaemia and I/R mainly inducing a decrease in cardiac cytoplasmic proteins. Alpha actin was the only protein showing an important increase likely reflecting a compensatory effect in order to restore myocardial tissue loss. Interestingly, we report that IPost-Co seems to revert and/or prevent 43% of such detected changes. Moreover, \textit{in silico} bioinformatic analysis showed a significant association between the differentially expressed cytoplasmic proteins and cell damage, being the most important canonical pathway involved the AhR signalling. We have found significant changes in Hsp90 which has been described to participate in the up-stream AhR signalling pathway. AhR resides in the cytosol associated with an Hsp90 dimer.\textsuperscript{30–32} Upon ligand binding, AhR translocates to the nucleus where it dimerizes with the ARNT protein (also known as Hif1β).\textsuperscript{32,33} It has been previously shown that reduction of Hsp90 level abrogates AhR function in a yeast model, probably because AhR is either improperly folded or destabilized in the absence of Hsp90.\textsuperscript{34} Therefore, we can hypothesize that the detected reduction in AhR and Hsp90 induced by IPost-Co leads to a down-regulation of the AhR signalling pathway eventually blocking intrinsic and extrinsic pro-apoptotic downstream effectors. In fact, we have confirmed this hypothesis by demonstrating the decrease in ARNT gene expression levels after IPost-Co. Moreover, our proteomic analysis reveals that I/R induces a decrease in β-tubulin levels which is

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\centering
\includegraphics[width=\textwidth]{ahr_pathway.png}
\caption{Proposed AhR signalling transduction pathway involved in IPost-Co-related cardiac protection. I, ischaemia; R, reperfusion; IPost-Co, ischaemic post-conditioning; AhR, aryl-hydrocarbon receptor; ARNT, aryl-hydrocarbon nuclear translocator; Hsp90, heat-shock protein 90.}
\end{figure}
reperfusion-triggered phenomena.\textsuperscript{3,4,2} IPost-Co antiapoptotic on the other hand, that our data support that apoptosis is mainly a part of the induced apoptosis in all the experimental groups. IPost-Co is generally considered as a post-conditioning treatment that started much before the induction of ischaemia (1.5 h) in all animals. Regarding the well-reported volatile anaesthetic-induced effect of isofluorane should not be considered since its administration started much before the induction of ischaemia (1.5 h) in all animals.\textsuperscript{2092}

AhR has also shown to promote Fas-mediated apoptosis in hepatocytes in the absence of exogenous AhR agonists.\textsuperscript{37} Here, we report that IPost-Co prevents reperfusion-triggered activation of both the death receptor (Fas/caspase-8) and mitochondrial apoptotic pathways eventually diminishing the detected amount of apoptotic cells in the jeopardized cardiac tissue as confirmed by TUNEL staining. Consequently, no changes were detected in the apoptotic marker Bcl-2, a counterbalance pathway of apoptosis execution. Hence, taking into consideration that, on the one hand, the final extent of infract size is not only determined by the severity of ischaemia\textsuperscript{38,39} but also by the pathological processes initiated at reperfusion that provoke additional tissue injury,\textsuperscript{5,40,41} and, on the other hand, that our data support that apoptosis is mainly a reperfusion-triggered phenomenon,\textsuperscript{3,4,2} IPost-Co antiapoptotic mechanisms should be regarded as a potential mechanism to partly explain the detected increase in salvaged myocardium. The quantitative contribution of apoptosis to final extent of infract size remains to be determined. Anversa’s group reported, in human myocardial samples obtained from the peri-infarcted region of patients who have recently suffered an myocardial infarction (MI), that around 12% of myocytes showed apoptotic signs emphasizing a potential detrimental effect of apoptosis execution on the final size of infarction.\textsuperscript{4} Intravenous administration of a caspase inhibitor\textsuperscript{43} or the transgene expression of the dominant-negative mutant of Fas-associated DD protein\textsuperscript{44} have shown to significantly attenuate cardiomyocyte apoptosis and reduce infract size. These observations not only suggest a link between cardiomyocyte apoptosis and the extent of myocardial infarction but that modulation of the apoptotic signalling (i.e. IPost-Co) may mediate a meaningful anatomic and functional cardiac rescue following I/R injury. Indeed, some indirect beneficial effect on ventricular function could also be expected from infract size limitation.\textsuperscript{45} Yet, it cannot be overlooked that IPost-Co may also confer an immediate cardiac protection by ameliorating myocardial blood flow contributing to the final infract size reduction.

Some potential limitations of our study deserve comments. As to the potential contribution of drug-related interferences in cardioprotection in our experimental setting, first, a post-conditioning effect of isofluorane should not be considered since its administration started much before the induction of ischaemia (1.5 h) in all animals. Regarding the well-reported volatile anaesthetic-induced pre-conditioning effect, this cannot be omitted, yet all animals were kept under the same conditions throughout the procedure. The same accounts to a potential protective effect of amiodarone on infract size and the prevention of ischaemic arrhythmias.\textsuperscript{46} Our observations must also be interpreted in the context of this study design. As such, we ought to provide further understanding on the underlying mechanisms and signal transduction behind IPost-Co in an animal model with human resemblance in order to provide new potential therapeutic targets. This study, however, does not consider important clinical situations found in most ST-elevation myocardial infarction patients. Indeed, the study includes juvenescent animals free from cardiovascular risk factors and hence, does not take into consideration the presence of certain co-morbidities and confounding factors (hyperlipaemia, hypertension, diabetes, age, etc.) as well as co-medications (adenosine, nitroglycerine, beta-blockers, statins, etc.) that have shown to interfere and/or attenuate IPost-Co cardioprotective effects.\textsuperscript{47} Finally, despite the small sample size, the behaviour was similar between the different animals within each group.

In conclusion, here we show that IPost-Co not only limits tissue cardiac damage but also improves cardiac contractility acutely after MI. In addition we report for the first time that one of the mechanisms of by which IPost-Co may limit apoptotic reperfusion injury involves the AhR signalling pathway.

Conflicting results exist as to the beneficial effect of IPost-Co in acute MI patients, some reporting a reduction on infarct size\textsuperscript{48} and others no protection.\textsuperscript{48} These conflicting results, which may derive from the presence of confounders (co-morbidities, co-medications, etc.), raise concerns about the clinical translation of the IPost-Co phenomenon. However, these results also highlight that there is still an important lack of studies addressed towards elucidating and understanding the molecular basis and the mechanisms triggered in vivo upon IPost-Co in large animal models with translational impact. These studies will help to decipher cardioprotective signalling pathways and targets in a model suitable for faster translational impact into the clinical arena. Importantly, the benefits of IPost-Co are not only limited to patients with acute coronary syndrome but also to patients undergoing cardiac surgery, cardiac arrest and transplantation.\textsuperscript{45,49}

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