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This is a pre print version of the following article:

Original Citation:

Availability:

This version hitst pa:v/a/ihldalb.lheandle.net/21318/201216-201-5-824T14:36:32

Published version:

DOI:10.1016/j.spc.2020.11.002

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09 August 2022

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This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

Penna C, Mognetti B, Tullio F, Gattullo D, Mancardi D, Moro F, Pagliaro P, Alloatti G. Post-ischaemic activation of kinases in the pre-conditioning-like cardioprotective effect of the platelet-activating factor. Acta Physiol (Oxf). 2009 Nov;197(3):175-85. doi: 10.1111/j.1748-1716.2009.02000.x

The definitive version is available at:

La versione definitiva è disponibile alla URL:

http://onlinelibrary.wiley.com/doi/10.1111/aps.2009.197.issue-3/issuetoc

Post-ischaemic activation of kinases in the preconditioning-like cardioprotective effect of the platelet activating factor

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Running Head: Signaling pathway of PAF cardioprotection

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Abstract

Purpose. Platelet activating factor (PAF) triggers cardiac preconditioning against ischemia/reperfusion injury. The actual protection of ischaemic-preconditioning occurs in the reperfusion phase. Therefore, we studied in this phase the kinases involved in PAF-induced preconditioning.

Methods. Langendorff-perfused rat hearts underwent 30-minutes ischaemia and 2-hours of reperfusion (Group 1, control). Before ischaemia, Group 2 hearts were perfused for 19-min with PAF ($2x10^{-11}$ M); Groups 3,4 and 5 hearts were co-infused during the initial 20-min of reperfusion, with the protein kinase C (PKC) inhibitor chelerythrine (5×10^{-6} M) or the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (5×10^{-5} M), and Atractyloside (2×10^{-5} M), a mitochondrial permeability transition pore (mPTP) opener, respectively. Phosphorylation of PKCε, PKB/Aκt, GSK-3β and ERK1/2 at the beginning of reperfusion was also checked.

Results. Left ventricular pressure and infarct size were determined. PAF-pretreatment reduced infarct size (33±4 *vs* 64±5 % of the area at risk of control hearts) and improved pressure recovery. PAF-pretreatment enhanced the phosphorylation/activation of PKCε, PKB/Aκt and the phosphorylation/inactivation of GSK-3β at reperfusion. Effects on ERK1/2 phosphorylation were not consistent. Infarct-sparing effect and post-ischaemic functional improvement induced by PAF-pretreatment were abolished by post-ischaemic infusion of either chelerythrine, LY294002, or Atractyloside. *Conclusions*. The cardioprotective effect exerted by PAF-pretreatment involves activation of PKC and PI3K in post-ischaemic phases, and might be mediated by prevention of mPTP opening in reperfusion *via* GSK-3β inactivation.

Key words: infarct size; phosphoinositide 3-kinase; protein kinase C; reperfusion injury.

Introduction

Reperfusion following a prolonged period of ischaemia leads to myocardial dysfunction and irreversible damages. A potent mean of protecting hearts from such ischaemia/reperfusion (I/R) injury is ischaemic preconditioning (IP), which consists in brief ischaemic periods interspersed by brief periods of reperfusion just before an infarcting ischemia (Murry *et al.* 1986, Liu *et al.* 1992; Khaliulin *et al.* 2007). IP reduces infarct size and post-ischaemic contractile dysfunction (Murry *et al.* 1986, Kimura *et al.* 1992, Gelpi *et al.* 2002).

Several agonists, such as adenosine, bradykinin and opioids (Yellon *et al.* 2003), have been proposed as triggers of the cardioprotective effect of IP. We have shown that low doses of *exogenous* platelet activating factor (PAF) are able to trigger preconditioning (Penna *et al.* 2005, 2008a). Importantly, *endogenous* PAF is involved in IP triggering (Penna *et al.* 2005). It has been shown that, besides to express PAF receptors, cardiac tissue is able to produce PAF. Specific binding sites for PAF were shown in several cell types within the heart, including cardiomyocytes and endothelial cells. Human cardiomyocytes express the same PAF receptor protein initially cloned from human leukocytes (Montrucchio et al 2000). PAF is released in the coronary effluent during reperfusion of isolated hearts undergoing I/R. Although the precise cellular source of PAF was not identified, likely candidates are endothelial cells and cardiomyocytes. Indeed, both cultured endothelial cells (Montrucchio et al 2000) and cardiomyocytes (Janero & Burghardt 1990) synthesize PAF after hypoxia. Yet, a very low increase in PAF levels occurs during exercise in normoalbuminuric diabetic patients (Cavallo-Perin *et al.* 2000) as well as during atrial pacing (Montrucchio *et al.* 2000 and references therein), both conditions known to be able to induce a preconditioning-like cardioprotective effect (reviewed in Pagliaro *et al.* 2001, Yellon *et al.* 2003). Human endothelial cells were found to produce PAF after stimulation by several inflammatory mediators, including tumour necrosis factor alpha and interleukins (Montrucchio *et al.* 2000), which have been seen to mimic IP (Smith *et al.* 2002). It may then be argued that low levels of PAF may represent a link between innate immunity and cardiac preconditioning. A cardioprotective role for

PAF *via* an S-nitrosylation mechanism has been recently confirmed by Leary *et al.* (2008). Similarly to ischeamic-preconditioning, the PAF-induced preconditioning signalling involves the *pre-ischaemic* activation of phosphoinositide 3-kinase (PI3K), protein kinase B (PKB/Akt), and protein kinase C (PKC) (Penna *et al.* 2005), as well as the activation of the mitochondrial ATP-dependent K^+ (mK_{ATP}) channels through a redox-sensible mechanism (Penna *et al.* 2008a).

Recent data suggest that reperfusion that follows infarcting ischemia may be a key moment of IP-induced protection (Hausenloy *et al.* 2002, Hausenloy & Yellon 2006a, 2006b, 2007, Murphy 2004a, 2004b). It seems that IP exerts its protective effect during reperfusion rather than ischaemic phase, and that activation and/or inhibition of multiple kinases are critical events which lead to the prevention of mitochondrial permeability transition pores (mPTP) opening during reperfusion (Hausenloy & Yellon 2007; Murphy 2004b; Cohen *et al.* 2006, Khaliulin *et al.* 2007). Also extracellular signal-regulated kinase (ERK1/2) has been involved in cardioprotection (Hausenloy & Yellon 2006b; Murphy 2004b; Yellon & Downey 2003). PI3K–Akt and/or ERK1/2 are part of pro-survival kinase cascades, which we refer to as the Reperfusion Injury Salvage Kinase (RISK) pathway. However, PAF given in reperfusion is detrimental (Montrucchio *et al.* 2000, Stangl *et al.* 2002, Stafforini *et al.* 2003).

Whether PAF-induced preconditioning also includes *post-ischaemic* kinase activation and prevention of mPTP opening is unknown. We hypothesize that low concentration of PAF given before the index ischaemia may lead to cardioprotection not only *via* the pre-ischaemic activation of kinases, but also *via* kinase activation and prevention of mPTP opening during reperfusion.

To verify the above hypothesis, we performed experiments in which we studied whether inhibitors of key kinases (i.e. PI3K or PKC) and mPTP opener (ATRA) given during post-ischaemic phase (i.e. during early reperfusion), can abrogate the protective effects of PAF-pretreatment. We verified whether PAF pre-treatment may lead to PKB/Aκt, PKCε, GSK-3β and ERK1/2 phosphorylation in post-ischaemic periods. Finally, we studied whether a mPTP opener given at reperfusion can prevent PAF-induced preconditioning.

Material and Methods

Animals

Male Wistar rats (n=59; body weight 460–540 g, five-six months old) 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).

Isolated heart preparation

The methods were similar to those previously described (Pagliaro *et al.* 2003, Penna *et al.* 2005, 2008a). In brief, animals were anaesthetized with urethane (1 g/kg i.p.), 10 min after heparin treatment the animal was decapitated, the chest opened and the heart rapidly excised. Isolated rat hearts were placed in ice-cold buffer solution, weighed, attached to the perfusion apparatus and retrogradely perfused 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), gassed with 95% O_2 and 5% CO_2 (pH=7.38). A constant flow was adjusted with a proper pump to obtain a typical coronary perfusion pressure of 80-85 mmHg during the initial part of stabilization. Thereafter, the same flow level (9±1 ml/min/g) was maintained throughout the experiment. A small hole in the left ventricular wall allowed drainage of the thebesian flow, and a polyvinyl-chloride balloon was placed into the left ventricle and connected to an electromanometer to record left ventricular pressure (LVP). The balloon was filled with saline to achieve an end-diastolic LVP (LVEDP) of 5 mmHg. Coronary perfusion pressure, coronary flow and LVP were monitored to asses the conditions of the preparations. The hearts were electrically paced at 280 bpm and kept in a temperature-controlled chamber (37° C).

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO), with the exception of reagents necessary to assess myocardial infarction, which were purchased by Merck (Whitehouse Station, NJ) and heparin, which was obtained from Roche (Milan, Italy).

Experimental protocols (Fig. 1)

After 20 min of stabilization, the hearts (n= 44) were randomly assigned to one of the eight treatment Groups described below and then subjected to a specific protocol, which included in all Groups a 30 min of global no-flow ischaemia followed by 120 min of reperfusion (Fig.1). Pacing was discontinued at the beginning of ischaemia and restarted after the third minute of reperfusion (Penna *et al.* 2005 and references therein).

Group 1 hearts (Control, n=7) were perfused with buffer solution only before and after the 30 min ischaemia.

Before ischaemia, *Group 2* (PAF_{pre}, n=7) hearts were perfused with buffer containing PAF (β acetyl-γ-O-alkyl-L-α phosphatidylcholine; $2x10^{-11}$ M) for 19 min, and then with buffer alone for 10 min; at this concentration, PAF did not cause any significant effect on cardiac performance (Penna *et al.* 2005, 2008a).

In *Group 3* (PAF_{pre}+CHE_{post}, n=5), and *Group 4* (PAF_{pre}+$LY294002_{post}$, n=5), hearts were perfused with PAF for 19 min, like group 2 hearts, but the first 20 min of reperfusion occurred in the presence of either chelerythrine (5×10^{-6} M) or LY294002 (5×10^{-5} M), respectively.

Group 5 (PAF_{pre}+$ATRA_{post}$, n=5) hearts were perfused for 19 min with PAF ($2x10^{-11}$ M) before ischaemia, but the first phase of reperfusion (20 min) occurred in the presence of the selective opener of mitochondrial transition pores, Atractyloside (ATRA, 20 μM), for 20 min. This concentration of ATRA has been previously shown to be non-toxic and to abolish the beneficial effects of pharmacological and ischaemic-preconditioning (Hausenloy *et al.* 2002, Park *et al. 2006*).

Fifteen control hearts (n = 5 for each group), subjected to I/R, were treated with CHE (CHE_{post}, *Group 6*) or LY294002 ($LY294002_{post}$, *Group 7*) and ATRA ($ATRA_{post}$, *Group 8*), during the first 20 min of reperfusion at the doses reported above (Hausenloy *et al.* 2002, Park *et al.* 2006).

The doses of the drugs used in the present study have been previously successfully used by other investigators and by us to antagonize the activity of kinases or to facilitate mPTP opening (Hausenloy *et al.* 2002, Penna *et al.* 2005, 2008a, Park *et al.* 2006 and references therein). The PAF

infusion timing and concentration were selected on the basis of our previous studies (Penna *et al.* 2005, 2008a).

Assessment of ventricular function

Changes in left ventricular end-diastolic pressure (LVEDP) and developed LVP (dLVP) values induced by the experimental manoeuvres were continuously monitored.

The increase in LVEDP was used as an index of the extent of contracture development. In fact, contracture development can be defined as an increase in intrachamber pressure of 4 mmHg above pre-ischaemic (baseline) LVEDP values (Pagliaro *et al.* 2003 and references therein]. Maximal recovery of dLVP during reperfusion was also compared with respective pre-ischaemic values.

Assessment of myocardial injury

To assess infarct areas, all hearts were rapidly removed from the perfusion apparatus at the end of reperfusion, and the left ventricle (LV) was dissected into 2–3 mm circumferential slices (e.g. Penna *et al.* 2005, Ma *et al.* 1999). 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 (F.T. or D.M.), who was not aware of the nature of the intervention. The weights of the necrotic and non-necrotic tissues were then determined, and the necrotic mass was expressed as a percentage of total left ventricular mass. Although the whole heart underwent normothermic ischaemia, we considered only the LV mass as area at risk, since in our experimental model the volume and the pre-load were kept constant for LV only.

Assessment of Kinases phosphorylation

Fifteen additional hearts (n=5 for each group) were treated as those of Control Group 1, PAF Group 2 and Sham-Group (no-ischemia, but only buffer perfusion for 75 min). In particular, according to Park *et al*. (2006), the hearts subjected to ischemia were removed from the perfusion system 7 minutes after the beginning of reperfusion (harrows of Fig. 1), and used to determine the total level and phosphorylation level of PKCε, PKB/Akt, ERK1/2 and GSK-3β. Sample preparation

and Western blot analysis were performed in duplicate as previously described (Penna *et al*. 2005). Briefly, blots were probed with primary rabbit polyclonal anti-PKCε (Upstate 06-991), anti-phospho-PKCε (Ser729, Upstate 06-821), anti-Akt (Cell Signaling 9272), anti-phospho-Akt (Ser473, Cell Signaling 9271), anti ERK1/2 (Cell Signaling 9107), anti-phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling 9106), anti-GSK-3β (Cell Signaling 9315) and anti-phospho-GSK-3β (Ser9, Cell Signaling 9336, USA) diluted according to manufacturer instructions. Blots were then probed with horseradish peroxidase-conjugated anti-rabbit antibody. Protein were detected using enhanced chemiluminescence ECL Western blotting detection reagent and bands were visualized through Kodak Image Station 440 CF. Only to confirm equal protein loading, blots were stripped with 0.4M NaOH and then re-blotted with an anti-actin antibody (Sigma 2066). Image analyses were performed by the Kodak 1D 3.5 software.

Statistical analysis

All data are expressed as means±S.E.M. One-Way ANOVA and Newman-Keuls Multiple Comparison Test (for post-ANOVA comparisons) were used to evaluate the statistical significance of the differences of the parameters between groups. One-way ANOVA has been used for the analysis of LVP data. In particular, comparison among area under the curves and comparison last time point were considered (Ludbrook 1994). A p value <0.05 was considered statistically significant.

Results

Isolated hearts

Cardiac weight (1467±29; range 1290–1770 mg, n =44) and the cardiac to body weight ratio (2.66±0.012; range 2.03–2.90 mg/g) were similar in the eight treatment groups. The risk area, i.e. LV mass, was also similar in all groups (LV weight was 917±19; range 570–1110 mg). The data show absence of inter-group differences about these three parameters.

Pre-ischaemic function.

Developed LVP (dLVP) and end-diastolic LVP (LVEDP) are reported in Table 1. The data show absence of inter-group differences about these two parameters. In particular, no significant differences of baseline cardiac function after stabilization and prior to ischaemia (i.e. after PAF-treatment or further buffer perfusion) were observed among the groups of hearts.

Contracture development during reperfusion (Fig 2)

Contracture has been suggested as a very good indicator of I/R injury in isolated rat hearts subjected to global ischaemia (Gelpi *et al.* 2002, Pagliaro *et al.* 2003, Penna *et al.* 2008b and references therein). The protocol of I/R induced in all groups an increase of LVEDP with respect to baseline (pre-ischaemic) values. However, the pre-treatment with PAF (PAF_{pre}, Group 2) significantly reduced LVEDP hastening with respect to Control Group 1 ($p<0.05$ between Group 1 and Group 2, Fig 2A and 2B, both area under the curve and last point). In groups in which the blocker was infused during the initial 20 min of reperfusion (i.e. $+CHE_{post}$ or $+LY294002_{post}$ or $+ATRA_{post}$,; Groups 3-5, Fig 2A), the LVEDP was significantly increased ($p< 0.05$ for all; both area under the curve and last point) with respect to PAF_{pre} alone (Group 2) (Fig 2A). Although not significant differences with respect to Control Group 1 were observed, a trend towards an increase in LVEDP was recorded in the additional Groups treated with the inhibitors alone (Groups 6-8, Fig 2B).

Contractile function during reperfusion (Fig. 3)

Post-ischaemic systolic dysfunction may be a result of stunning and necrosis (Gelpi *et al.* 2002; Penna *et al.* 2008b). Post-ischaemic recovery of systolic function was evaluated as % of dLVP recovery during reperfusion. As can be seen in Fig. 3A and 3B, 30 min ischaemia depressed contractile function in all groups as compared to baseline values.

PAF pretreatment (Group 2) reduced contractile dysfunction with respect to other Groups ($p<0.05$ between Group 1 and other Groups, both area under the curve and last point, Fig. 3A). In particular, at the end of reperfusion, dLVP decreased by about -45% with respect to baseline condition in Group 2, whereas this reduction was by about -75% in Control Group 1.

The treatment with inhibitor of PKC (+CHE_{post}, Group 3) or PI3K (+$LY294002_{post}$, Group 4) as well as with mPTP agonist (+$ATRA_{post}$, Group 5) during reperfusion, abolished the cardioprotective effect of PAF-pretreatment (Fig 3A). In the additional Groups (Groups 6-8, Fig 3B), the infusion of the inhibitor alone (pre- or post-ischaemia) did not cause significant alterations with respect to Control Group 1 (p = Not Significant for all, both area under the curve and last point). Developed LVP recovery of both CHE_{post}, and $LY294002_{post}$ was also not significantly different from PAF pretreated hearts (Fig 3B).

Infarct size (Fig 4)

In Group 1 hearts, the infarct size measured at the end of reperfusion was 64±5% of the area at risk. Group 2 hearts (PAF_{pre}) showed a significantly ($p<0.01$) reduced infarct size (33±4% of the area at risk) respect to Control Group 1 hearts. In Group 3 and 4, the infusion of PKC antagonist (+CHE_{post}) or PI3K inhibitor (+$LY294002_{post}$) in the first phase of reperfusion (20 min) blocked the cardioprotective effect of PAF-pretreatment (63±2% and 61±4% of area at risk respectively; p= non significant *vs* Group 1, and $p<0.05$ *vs* Group 2 for both) (Fig 4 A).The administration of the mPTP opener (+$ATRA_{post}$) during the first 20 min of reperfusion also completely abolished the cardioprotective effect induced by PAF pre-treatment (64±11% of the area at risk; p= non significant *vs* Group 1, and $p<0.05$ *vs* Group 2).

In groups 6-8 (Fig 4B), in which the inhibitor only was infused in the post-ischaemic phase, the infarct size (70±3% and 68±7% and 64±6% of area at risk, respectively) was not statistically different from that observed in the control Group 1 (p= non significant *vs* Group 1, and $p<0.05$ *vs* Group 2 for all). The data obtained for groups 6-8 are in agreement with those previously reported by other authors who used these agents (Penna *et al.* 2005 and the reference therein, Hausenloy *et al.* 2002, Park *et al.* 2006).

Western blotting (Fig 5)

Densitometrical analysis of the scanned blots, presented in Fig. 5, showed that phospho-Aκt/total Aκt ratioincreased after I/R only compared to sham level, and increased even more in

PAF-pretreated hearts that underwent I/R. Yet, in hearts subjected to I/R only the levels of phospho-PKCε and phospho-GSK-3β decrease. Such a decrease is reversed by pre-treatment with PAF. Also phospho-ERK1/2 decreased after I/R, a decrease which did not reach statistical significance in hearts pretreated with PAF. In fact p-ERK is not consistently reduced in pretreated hearts. These findings suggested a link between the level of phosphorylation of PKB/Akt (upstream) and the other two downstream kinases during reperfusion (*i.e.* activated phospho-Akt may directly or indirectly lead to PKCε and GSK-3β phosphorylation). ERK is a parallel harm of the RISK pathway which can or cannot be activated in preconditioning (Yellon & Downey 2003).

Discussion

This study indicates that cardioprotection triggered by PAF-pretreatment depends on the activation of kinases after ischaemia. In fact, kinase antagonists given in reperfusion abolishes PAF-induced protection. We previously showed that PAF is an endogenous activator in ischaemic preconditioning along with other activators of Gq-coupled receptors (Penna et al. 2005). The present study suggests that PAF-induced cardioprotection is mediated by activation of PI3K and by enhanced phosphorylation/activation of PKB/Akt, as well as by preserved phosphorylation/inactivation of GSK-3β in the post-ischaemic phase. In particular, we show for the first time that the levels of phospho-Akt are kept higher in the post-ischaemic phase, both in PAF-pretreated and non-treated hearts. Yet, in PAF-pretreated hearts the phospho-Akt level in reperfusion is significantly higher than that of non-treated hearts. On the other hand, the levels of phospho-PKCε and phospho-GSK-3β tend to decrease in post-ischaemic period. Such a decrease is prevented by PAF-pretreatment. This confirms that GSK-3β can be phosphorylated/inhibited by the Ser/Thr kinases, such as PKB/Akt and PKCε. Yet, it seems that during reperfusion a preservation of the pre-ischaemic level of phospho-GSK-3β is enough to reduce cell death. Phospho-Akt may also stimulate the nitric oxide synthase (NOS) (Yellon et al. 2003), the activity of which plays an important role in PAF-induced cardioprotection (Leary et al. 2008). Although we cannot exclude

that endothelium play a relevant role in PAF-dependent protection, several data suggest a direct effect of PAF on cardiomyocytes (Montrucchio *et al.* 2000 and references therein). Besides to confirm the cardioprotective effect of PAF on the isolated heart undergoing I/R, the recent paper by Leary *et al.* (2008) demonstrated indeed that PAF exerts a beneficial effect also on isolated cardiomyocytes (i.e. in the absence of endothelium), *via* a NO-dependent, S-nitrosylation mechanism.

The ERK1/2 cascade, when activated in the setting of ischaemia–reperfusion, can mediate cellular protection. However, ERK is not consistently part of preconditioning's signaling (Yellon & Downey 2003), as again shown in this study. We previously showed that PAF preconditioning involves PKC and PI3K (Penna et al. 2005), as well as mK_{ATP} and ROS (Penna et al. 2008a) in the pre-ischaemic phase. In fact the co-infusion with an antioxidant (N-Acetylcysteine) abolished completely the cardioprotective effect of PAF (Penna et al. 2008a).

Data of the present study suggest that PAF-treatment prior to ischaemia somehow (i.e. with the involvement of mK_{ATP}, ROS, PKB/Akt and PKCε) activates a memory function that results in a re-activation of PKB/Akt, PI3K and PKC at reperfusion. This reactivation represents pivotal steps of the so-called RISK pathway and may allow prevention of mPTP opening at reperfusion (Yellon et al. 2003, Hausenloy et al. 2006a, 2006b, 2007). In fact, these authors suggest that in the reperfusion phase of preconditioned hearts PKB/Akt may be upstream to PKCε, which in turn may be upstream to GSK-3β.

Activation of the PI3K/Akt/NOS signalling pathway has also been demonstrated to provide cardioprotection against various stressors by preserving mitochondrial integrity and function. This pathway signalling may converge on the prevention of mPTP opening to lead to cardioprotection (Murphy 2004a, Gustafsson et al. 2008). Opening of the mPTP is predominantly associated with necrotic cell death, most likely in those cells that have already sustained injury during ischemia, thus accelerating cell death. It is likely that conditions occurring during early reperfusion, rather than during ischaemia, favour the formation of opened-mPTP, and that inhibition of pore opening in

reperfusion protects against cell death. Preconditioning may also provide continuing protection during reperfusion by preventing a cascade of mPTP-induced ROS production followed by further mPTP opening. This phase of protection may involve survival kinase pathways, such as Akt and GSK-3β, either increasing ROS removal or reducing mitochondrial ROS production (Halestrap et al. 2007).

A pre- or post-ischaemic activation of PKC in the preconditioning scenario is considered protective by the majority of the authors (e.g. Yellon et al., 2003, Penna et al. 2005, Murphy 2004b). It has been suggested that activated PKC forms a complex with components of mPTP (Murphy 2004b) and may represent an additional mechanism for the closure of this pore in reperfusion. In fact, many authors (e.g. Murphy 2004a, Griffiths et al. 1995, Juhaszova et al. 2004), report that the opening of mPTP usually occurs in reperfusion when phosphorylated GSK-3β is reduced. So that we can argue that pre-treatment with PAF may limit mPTP opening and cell death in reperfusion. Since GSK-3β is downstream to both PKC and PI3K (Juhaszova et al. 2004), we suggest that GSK-3β phosphorylation/inhibition by the cascade activated by the other two kinases may positively affect ischaemic tolerance, preventing mPTP opening. Here, we demonstrated that PAF-pretreatment may modulate the activity/phosphorylation of GSK-3β in post-ischaemic phase in cardiac tissue. As a matter of fact, we were also able to prevent PAF-protective effects by favouring the opening of mPTP with ATRA given in reperfusion. Data suggest that PAF-pretreatment impacts mPTP opening to affect protection.

Limitation of the study

In the present study, the involvement of RISK activation is based on Western blotting and the use of pharmacological inhibitors. However, some methodological questions emerge: harvesting the whole ventricle for biochemical analysis permits neither the simultaneous determination of infarct size or infarct delineation nor the determination of baseline values from the same heart. Therefore, the samples are always a mixture of salvaged and necrotic tissue. Involvement of a kinase is usually measured as the degree of its phosphorylation; however, phosphorylation status could be dependent

on availability of ATP, which is restricted after ischemia. Thus it is not easy to predict the sequence of a signalling cascade based on Western blot measurements at one time point. However, the very nature of the endpoint of myocardial protection – reduction in infarct size – implies that with successful protection, there is more viable tissue and, since phosphorylation of a kinase occurs only in viable tissue, greater phosphorylation of a specific kinase may just reflect more viable tissue (see Skyschally et al., 2008).

All inhibitors used to study RISK activation have, apart from their specific properties, also other less specific effects on cell function This is particularly true in the case of ATRA, a pharmacological mPTP opener, used in the present study to block PAF preconditioning, We used ATRA at a dose that, in spite of a tendency to increase LVEDP during reperfusion, does not induce *per se* additional necrosis (see also Hausenloy *et al*. 2002). However, ATRA effects do not implicate a definitive role for PAF in the modulation of mPTP. In fact, interventions that pharmacologically open the mPTP would be expected to abrogate cardiac protection induced by any form of cardioprotection, though used at a no-lethal dose. Therefore, the use of ATRA does not precisely address the role of mPTP in PAF-protection, but shows that mPTP opening is responsible for I/R tissue injury, which may be central in PAF prevention of cardiac damages. To definitely assess whether PAF involves mPTP closure as a downstream mechanism, future studies should directly test whether this agent impact mPTP opening in response to stress. Finally, since the drug does not increase infarction in unprotected tissue (Hausenloy *et al*. 2002 and present study) it may be assumed that it reverses the protection in protected tissue by opening the pores that protection was keeping closed. In fact it has been suggested that the opening of mPTP is the final step of damages already initiated in ischaemic phase (Murphy 2004b, Gustafsson *et al.* 2008).

Previous studies indicated that PAF administered at high concentrations (micromolar range) are detrimental for cardiac function (Montrucchio *et al*. 2000, Stangl *et al*. 2002, Stafforini *et al*. 2003). In particular at high concentrations PAF is able to modulate the activity of GSK-3β in other non-ischaemic tissues. In granule neurons (Maggirwar *et al*. 1999, Tong *et al*. 2001), PAF has been

implicated as a mediator able to enhance the activity of GSK-3β, thus exerting deleterious effects. We can argue that PAF, ROS and other agents involved in cardioprotection (Yellon et al. 2003, Murphy 2004a,b, 2005) may exert dual effects, being able to be protective or deleterious, depending on several factors (e.g. timing, concentration of the agents and/or the cell system/compartmentalization). It must be taken into account that, besides to confirm the preconditioning-like effect of PAF, a recent paper by Leary *et al.* (2008) suggested that the nitric oxide-induced S-nitrosylation of Ca^{2+}-handling proteins, such as L-type Ca^{2+} channels, may be an alternative pathway to explain PAF protection. Further studies are required to investigate these last points.

In conclusion: it has been previously reported that endogenous PAF participates to IP triggering and that low doses of PAF are able to induce cardioprotection similar to IP (Penna *et al.* 2005, 2008a, Leary *et al.* 2008). Besides to confirm these data, here we give new insights into the intracellular pathways involved in the action of PAF. We show here that also the activation of PI3K/Akt and PKC is a fundamental mechanism for PAF cardioprotection during post-ischaemic period, in PAF-preconditioned hearts. ERK cascade seems not consistently involved. Since the mPTP opener ATRA given in reperfusion abolishes PAF-induced protection, we can argue that the effect of PAF converges on this pore.

Our data emphasize the potential importance of a moderate release of PAF, such that occurring during exercise, as an attempt of the cardiovascular system to protect itself against I/R damages.

Acknowledgments

The authors are supported by Regione Piemonte, PRIN, and University of Turin. We Thank DrMaria-Giulia Perrelli and Dr Annalisa Merlino for technical assistance.

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Legends of Figures

Fig. 1. Experimental protocols

Before ischemia, hearts were divided into 8 groups. Control hearts (*Group* 1) were perfused with buffer alone; *Group 2* hearts were treated with platelet-activating factor PAF, ($2x10^{-11}$M); in *Group 3* (PAF_{pre}+CHE_{post}, n=5), *Group 4* (PAF_{pre}+$LY294002_{post}$, n=5) and *Group 5* ((PAF_{pre}+$ATRA_{post}$, n=5) hearts were perfused with PAF for 19 min, like group 2 hearts, but the first 20 min of reperfusion occurred in the presence of either chelerythrine (CHE) or LY294002 or Atractyloside (ATRA) respectively. Groups 6-8 are control groups in which the antagonists were infused without PAF. Arrows indicate the moment in which Western blot analysis were performed.

Fig. 2. Left ventricular end diastolic pressure (LVEDP) during ischemia and reperfusion.

Time 0 marks the onset of 120 min reperfusion which follows 30 min global ischemia (beginning at time -30). Data are expressed as mmHg. Panel A Groups 1-5; Panel B Groups 1,2 and 6-8. In Panel B groups 1 and 2 are reported for comparative purpose. CHE = PKC antagonist; LY294002 = PI3K antagonist; ATRA = mPTP opener. Data are means ±SEM. **= $p < 0.05$ with respect to Control group last point; c= $p < 0.05$ with respect to PAF_{pre} group last point; #=$p <0.05$ with respect to the indicated areas under the curve.*

Fig. 3. Developed left ventricular pressure (LVP) during ischemia and reperfusion.

Time 0 marks the onset of 120 min reperfusion which follows 30 min global ischemia (beginning at time -30). Data are expressed as % change from pre-ischaemic baseline. Panel A Groups 1-5; Panel B Groups 1,2 and 6-8. In Panel B groups 1 and 2 are reported for comparative purpose. CHE = PKC antagonist; LY294002 = PI3K antagonist; ATRA = mPTP opener. Data are means ±SEM. **= $p < 0.05$ with respect to Control group last point; c= $p < 0.05$ with respect to PAF_{pre} group last point; #=$p <0.05$ with respect to the indicated areas under the curve.*

Fig. 4. Role of kinases and mPTP in cardioprotective effect of PAF.

Figure represents the infarct size at the end of reperfusion (120 min), expressed as percentage of left ventricle (LV) mass. Panel A Groups 1-5; Panel B Groups 1,2 and 6-8. In Panel B

groups 1 and 2 are reported for comparative purpose. CHE = PKC antagonist; LY294002 = PI3K antagonist; ATRA = mPTP opener. Data are means ±SEM. **$p < 0.01$ *vs* Control. # $p < 0.05$ *vs* PAF. Acronyms as in the text.

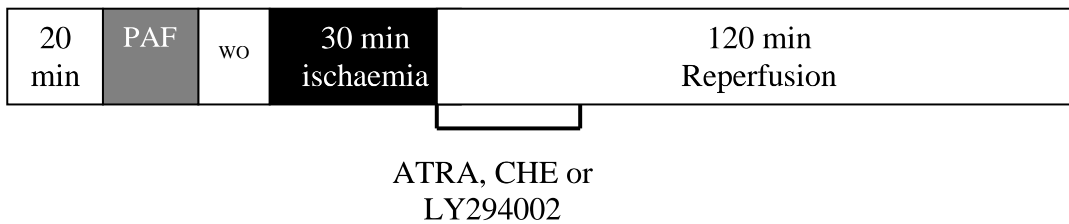
Fig.5 Western Blot analysis

Western blot analysis relative to the phosphorylation of kinases induced by PAF pretreatment and/or index ischaemia. Panels from top to bottom represents the normalized data and representative bands of Akt (p-Akt), PKCε (p-PKCε),GSK-3β (p-GSK-3β) and ERK1/2 (p-ERK1/2). Data are normalized with respect to the mean value of densitometric level of Sham Group hearts (no ischaemia). Samples were collected 7 min after ischaemia from hearts pre-treated with PAF (PAF+I/R) or untreated (I/R). In Sham group, samples were collected 75 min after buffer perfusion only. *$p < 0.05$ vs Control, # $p < 0.05$ vs Ischemia only. For acronyms and further explanation see text.

Group 1, Control

Group 2, PAF$_{pre}$

Groups 3, 4 and 5, PAF$_{pre}$+Antagonist$_{post}$ (ATRA, CHE or LY294002)



Groups 6, 7 and 8, Antagonist$_{post}$ only (ATRA, CHE or LY294002)

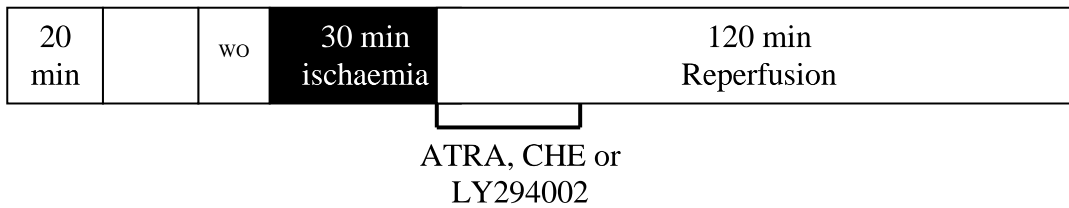
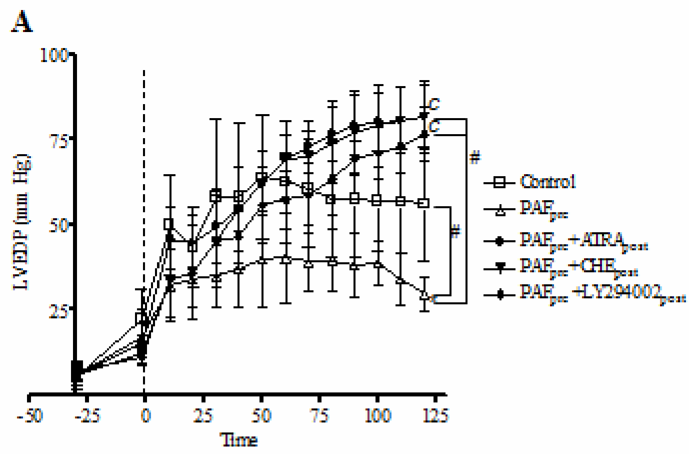


Fig.1



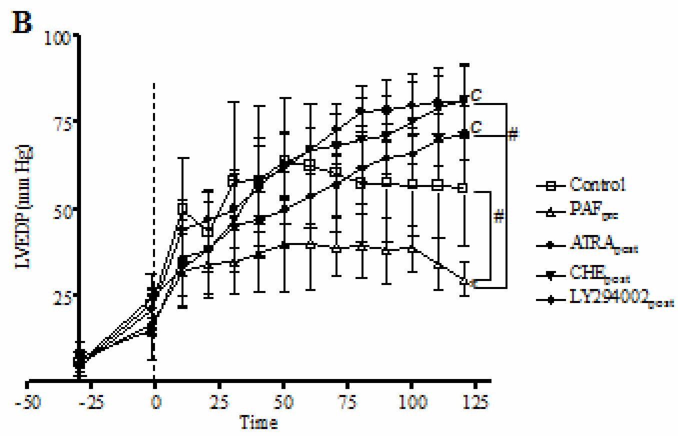
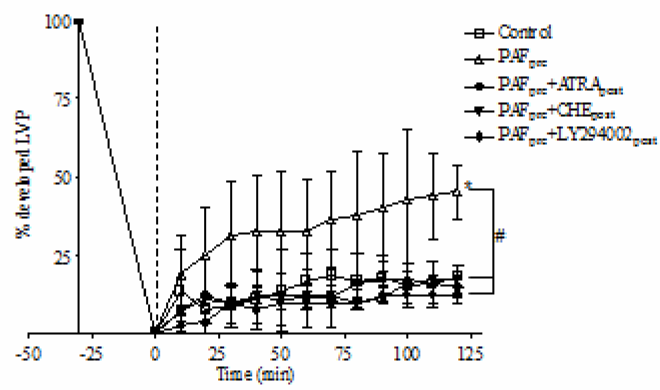


Fig.2



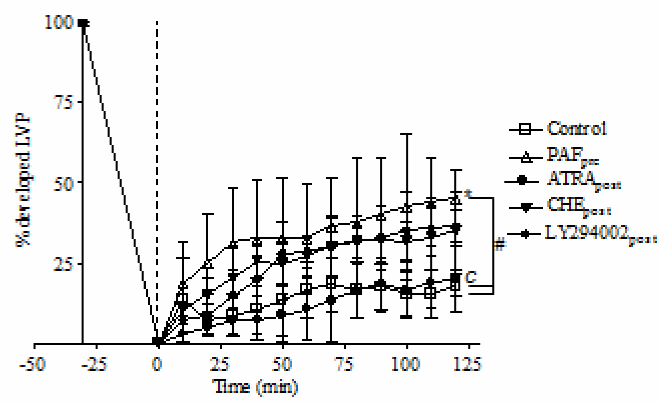
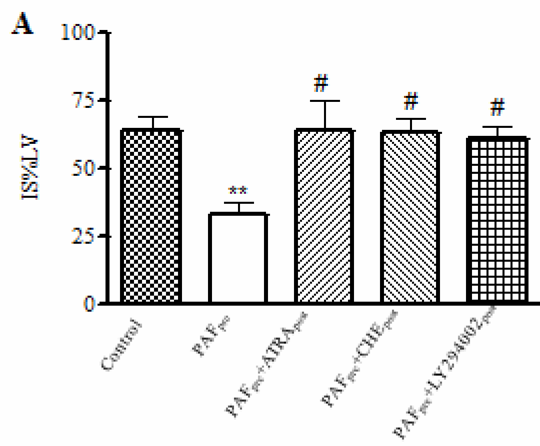


Fig.3



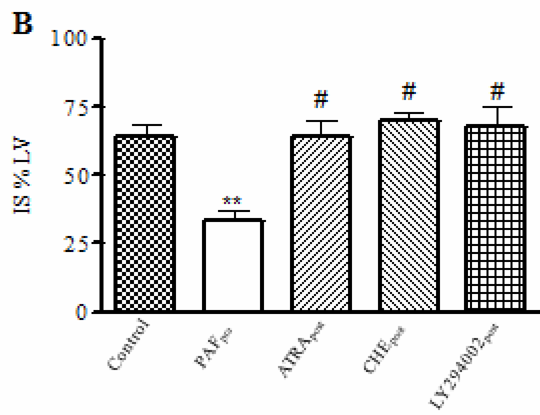


Fig.4

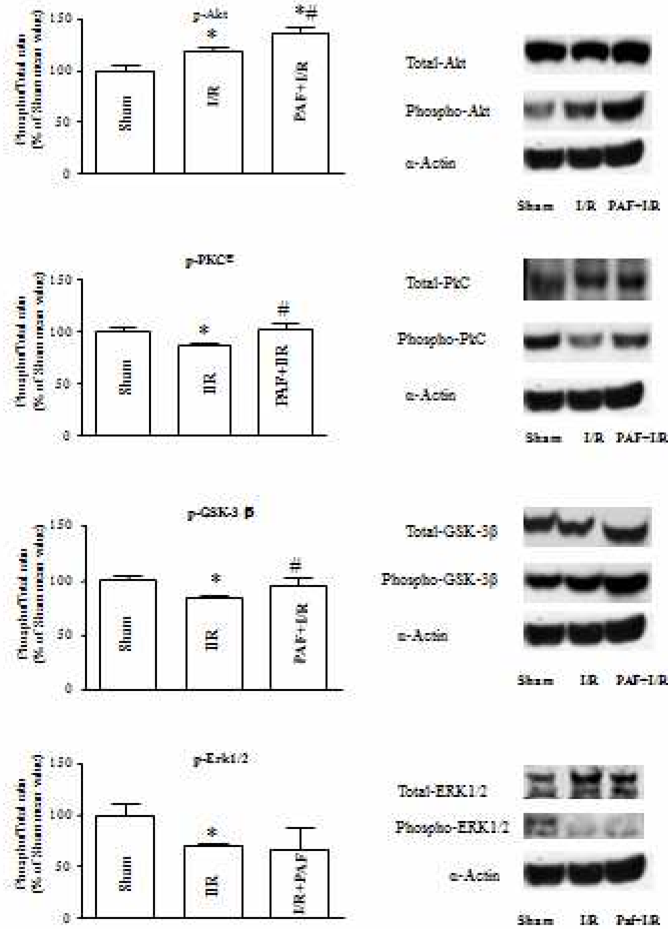


Fig.5

	Stabilization		Before Ischemia		End Reperfusion	
Group	dLVP **mmHg**	LVEDP **mmHg**	dLVP **mmHg**	LVEDP **mmHg**	dLVP **mmHg**	LVEDP **mmHg**
Control (Group 1)	75±5	5±3	74±7	5±3	15±2 *	56±16 *
PAF_{pre} (Group 2)	79±3	5±3	81±7	6±3	36±4 *#	30±5 *#
PAF_{pre}+ CHE_{post} (Group 3)	80±2	5±2	83±2	6±2	15±2 *	81±9 *
PAF_{pre}+$LY294002_{post}$ (Group 4)	81±5	6±2	85±4	7±3	12±2 *	76±9 *
PAF_{pre}+$ATRA_{post}$ (Group 5)	77±7	5±2	78±2	6±2	10±4 *	81±10 *
CHE_{post} (Group 6)	78±6	5±1	77±7	5±2	25±5 *	78±9 *
$LY294002_{post}$ (Group 7)	78±5	6±1	79±3	7±1	27±4 *	74±6 *
$ATRA_{post}$ (Group 5)	72±5	5±2	75±5	6±1	16±2 *	78±10 *

Table 1. Functional parameters during stabilization, before ischemia and at the end of reperfusion. dLVP= developed left ventricular pressure; LVEDP = Left ventricular and diastolic pressure. Other acronyms as in the text.
* $p < 0.05$ vs Before Ischemia; # $p < 0.05$ vs Control group.