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Phosphoinositide 3-kinase γ -deficient hearts are protected from the PAF-dependent depression of cardiac contractility

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Abstract

Objectives: Following an ischemic insult, cardiac contractile recovery might be perturbed by the release of autacoids, like platelet-activating factor (PAF), that depress heart function by acting through G protein-coupled receptors (GPCRs). The signaling events downstream the PAF receptor that lead to the negative inotropic effect are still obscure. We thus investigated whether the GPCR-activated phosphoinositide 3-kinase γ (PI3K γ) could play a role in the cardiac response to PAF. **Methods:** The negative inotropic effect of PAF was studied *ex vivo*, in isolated electrically driven atria and in Langendorff-perfused whole hearts derived from wild-type and PI3K γ -null mice. Posts ischemic recovery of contractility was analyzed in normal and mutant whole hearts subjected to 30 min of ischemia and 40 min of reperfusion in the presence or absence of a PAF receptor antagonist. **Results:** While wild-type hearts stimulated with PAF showed increased nitric oxide (NO) production and a consequent decreased cardiac contractility, PI3K γ -null hearts displayed reduced phosphorylation of nitric oxide synthase 3 (NOS3), blunted nitric oxide production and a complete protection from the PAF-induced negative inotropism. In addition, Langendorff-perfused PI3K γ -null hearts showed a better contractile recovery after ischemia/reperfusion, a condition where PAF is known to be an important player in depressing contractility. In agreement with a role of PI3K γ in this PAF-mediated signaling, posts ischemic contractile recovery in PI3K γ -null mice appeared overlapping with that of normal hearts treated with the PAF receptor antagonist WEB 2170. **Conclusion:** These data indicate a novel PAF-dependent signaling pathway that, involving PI3K γ and NOS3, contributes to posts ischemic contractile depression.

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Keywords: Signal transduction; Inotropic agents; Ischemia; Nitric oxide

1. Introduction

In the heart, homeostatic regulation and stress responses are mainly controlled by the extracellular signals transduced by G protein-coupled receptors (GPCRs). Upon activation, these receptors trigger the production of second messengers, such as the lipid phosphatidylinositol (3,4,5)-trisphosphate

(PtdIns(3,4,5)P₃) [1]. Class I phosphatidylinositol 3-kinase selectively phosphorylate the 3'-OH residue of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂) to form PtdIns(3,4,5)P₃, which serves as a docking site for cytoplasmic proteins involved in multiple cellular processes such as survival, proliferation, cytoskeletal remodeling and membrane trafficking [2].

Class I PI3Ks consist of dimers that are classified in two subfamilies depending on their composition and mechanism of activation (for a review, see Ref. [3]). PI3Ks of the first subgroup (class IA) are formed by the catalytic subunit associated with a p85-like regulatory protein docking to

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phosphorylated tyrosines in YXXM motives. The other group is instead characterized by the interaction of the catalytic subunit with an adaptor called p101 and by the activation through $\beta\gamma$ subunits of trimeric G proteins (class IB). The only class IB member, phosphoinositide 3-kinase γ (PI3K γ), is highly expressed in white blood cells but can be detected at lower levels in the cardiovascular system [4–6]. We and others recently reported that PI3K γ -deficient mice were viable and fertile, but displayed defective GPCR responses to chemoattractants in leukocytes [7–9], to ADP in platelets [10] and to adenosine in mast cells [11]. On the other hand, little is known about the role of PI3K γ in the cardiovascular system [12]. Interestingly, PI3K γ has been found to be expressed in bovine aortic endothelial cells and to signal under shear stress to mitogen activated protein kinase (MAPK) [13]. PI3K γ also appears to control angiotensin-II-dependent L-type calcium channel activation in vascular smooth muscle cells [5]. Moreover, PI3K γ has been shown to be present in cultured cardiomyocytes and to mediate the ATP-dependent generation of autonomic calcium oscillations by modulating Tec and Fyn kinases, and phospholipase C γ (PLC γ) [4].

In agreement with these findings, we recently showed that PI3K γ acts as a negative regulator of myocardial contractility [14]. This suggested that the lack of PI3K γ might lead to improved heart function in response to GPCR agonists playing maladaptive roles in cardiac pathologies. Here, we provide the evidence that PI3K γ -null hearts show improved contractility in response to platelet-activating factor (PAF), a GPCR agonist released during ischemia and involved in depressing postischemic cardiac function.

2. Methods

2.1. Mice

PI3K γ -null mice were obtained as previously described [7]. All experiments were performed with female 8-week-old wild-type and PI3K $\gamma^{-/-}$ mice; both mutant and control animals were inbred 129 sv. Mice were anesthetized with avertin and sacrificed by cervical dislocation. Animal experiments were carried out in compliance with institutional guidelines as well as with Italian and Swiss government regulations.

2.2. Analysis of contractility in isolated cardiac muscle preparations

After sacrifice, heart was rapidly removed, and atria were dissected free under microscopic control. Tissue preparations were superfused with a modified Tyrode solution (154 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5.5 mM D-glucose, 5 mM HEPES, adjusted to pH 7.36 with NaOH and gassed with 100% O₂). Cardiac muscles were equilibrated in modified Tyrode solution for

at least 30 min before each experiment, and experiments were conducted at 37 °C. Atria were driven at a frequency of 240 beats/min with a pair of electrodes connected to a 302 T Anapulse Stimulator via a 305-R Stimulus Isolator (W. P. Instruments, New Haven, CT, USA) operating in constant current mode. Isometric twitches were measured by a Harvard Isometric Transducer 60-2997 and were recorded and analyzed using a custom-made Labview based VI (National Instruments, USA) on an Apple Power-Mac. Endocardial endothelium was removed as previously described [15]. Briefly, cardiac preparations were treated with Tyrode solution containing 0.5% Triton X-100 for 1 or 2 s and then recovered in Triton-free Tyrode solution for 30 min.

2.3. PAF-induced Akt/PKB and nitric oxide synthase 3 (NOS3) phosphorylation and NO production

Isolated mouse hearts were washed with PBS, weighed and incubated in PBS–BSA 0.25% for 2 min in ice. The hearts were transferred at 37 °C and then stimulated 10 min with PAF 1 μ M or PBS–BSA 0.25%. Immediately thereafter, hearts were frozen in liquid nitrogen.

For immunodetection of proteins, frozen heart tissue samples were homogenized by sonication in 50 mM Tris pH 7.5, 150 mM NaCl containing 1% NP-40 for immunoprecipitation and 1% SDS for Western blot. Immunoprecipitation of PI3K γ was performed using a rabbit polyclonal antibody and detected with a monoclonal antibody (both kindly provided by Reinhard Wetzker, Jena, Germany). Western blots for analysis of phosphorylation were probed overnight at 4 °C with polyclonal antibodies that recognize phosphorylated Akt (Ser-473), phosphorylated NOS3 (Ser-1177), total Akt or a monoclonal antibody recognizing total NOS3 (all from Cell Signaling Technology, Beverly, MA).

For the evaluation of NO production, frozen hearts were rapidly minced, placed in ice-cold PBS (total volume 1.5 ml) and immediately homogenized. Samples were centrifuged at 100 000 \times g for 20 min, and the supernatants were stored at –80 °C. Cardiac NO content was measured with a fluorimetric assay kit (Cayman, Ann Arbor, MI) that detects nitrite/nitrate NO degradation products following the manufacturer's instructions. Assays were repeated three times with reproducible results. For the analysis of pertussis toxin effects on PAF-dependent NO production, mice were administered either pertussis toxin 30 μ g/kg (Sigma) in saline or saline alone by intraperitoneal injection. This protocol is known to specifically inactivate inhibitory guanine nucleotide proteins (G_i and G_o) in cardiac tissue [16]. Hearts were collected 24 h later and treated as above.

2.4. Model of myocardial ischemia/reperfusion injury

The isolated mouse hearts were dissected and weighed (weight appeared similar in the two genotypes: 232.5 \pm 13.3

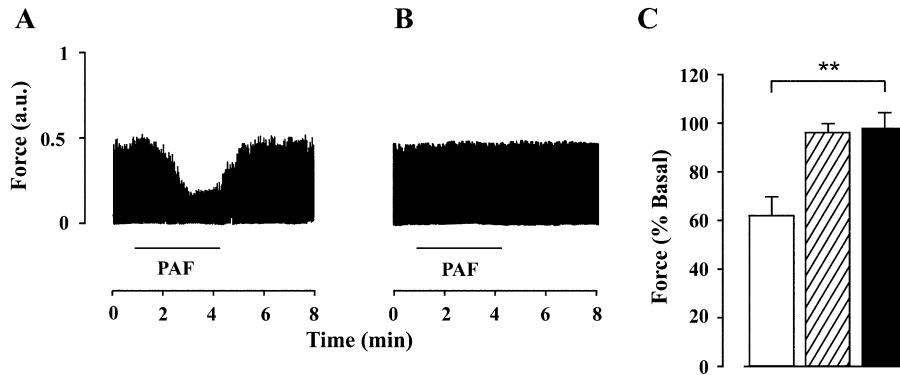


Fig. 1. PAF-dependent effects on cardiac contractility. (A and B) Representative traces of the contractile force (arbitrary units) generated by wild-type (A) and $PI3K\gamma^{-/-}$ (B) electrically paced (240 beats/min) atrial muscles exposed to $1\ \mu\text{M}$ PAF. (C) Summary of PAF ($1\ \mu\text{M}$)-mediated effects on contractility of wild-type (white bar; $n=6$) and $PI3K\gamma^{-/-}$ (black bar; $n=5$) atria. The hatched bar corresponds to wild-type samples ($n=3$) treated with PAF in the presence of the PAF receptor antagonist WEB 2170 ($3\ \mu\text{M}$). Bars represent force of contraction in % of the basal value. Baseline values of contractile force were 30.8 ± 4.3 and 34.0 ± 5.4 mg for wild-type and mutant atria, respectively. Treatment of wild-type isolated atria with WEB 2170 did not significantly change baseline contractility. Statistical difference of wild-type versus mutant as determined by ANOVA with Newmann–Keuls post hoc test: $**P < 0.01$.

and 253.0 ± 16.4 mg for wild-type and mutant hearts, respectively). Hearts were mounted on a Langendorff perfusion apparatus, and perfused at a constant flow rate (12 ml/min/g of wet weight) using a peristaltic pump with modified Tyrode solution warmed to $37\ ^\circ\text{C}$ and gassed with 100% O_2 . The force of contraction was monitored through a hook attached to the LV apex under 1 g of preload. Pacing electrodes were inserted in the right ventricle and pacing was adapted to maintain a heart rate of 240 beats/min. After stabilization, the hearts were subjected to 30 min ischemia with coronary flow reduced to 3% of the control preischemic value, followed by 40 min of reperfusion at the original flow rate. Controls were perfused continuously for the same amount of time. Left ventricular contractile force was measured with a force–displacement transducer (Harvard Isometric Transducer 60-2997) attached to the apex through a metal hook. The ventricular force signal was digitally processed to yield peak systolic contractile force, first derivative of contractile force development or relaxation over time ($\pm dF/dt$). In experiments with the PAF receptor antagonist, hearts were perfused for 10 min before ischemia and during the ischemia/reperfusion with Tyrode solution containing $3\ \mu\text{M}$ WEB 2170 (Boehringer Ingelheim, Germany). During the 10 min of pretreatment, this concentration of antagonist did not significantly alter cardiac performance.

2.5. Statistical analysis

Data are expressed as the mean \pm S.E.M. Comparisons used unpaired t test for differences between wild-type and $PI3K\gamma$ -deficient mice, paired t test, repeated-measures two-way ANOVA followed by Bonferroni post hoc test or one way ANOVA followed by Newmann–Keuls post hoc test as appropriate. P value of <0.05 was assigned statistical significance.

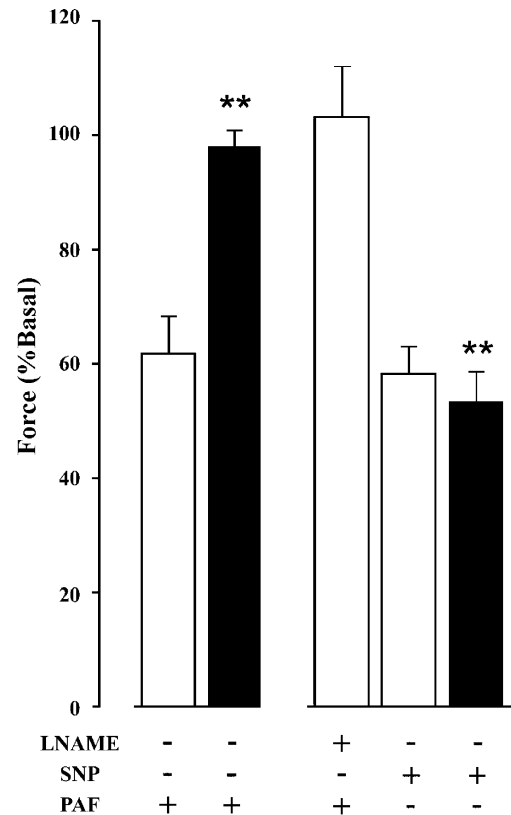


Fig. 2. Effects of PAF-mediated NO production on cardiac inotropism. (a) Analysis of the role of NO in the PAF-dependent induction of negative inotropism in electrically paced (240 beats/min) atrial muscles. Bars (white, wild-type; black, $PI3K\gamma^{-/-}$) represent force of contraction of electrically paced (240 beats/min) atrial muscles in % of the basal value after stimulation with $1\ \mu\text{M}$ PAF in the presence or absence of the NOS inhibitor L-NAME ($1\ \text{mM}$; $n=4$) and of the NO donor sodium nitroprusside (SNP; $1\ \mu\text{M}$; $n=4$ for both genotypes). Contractile force baseline values were 31.7 ± 3.2 and 33.8 ± 4.3 mg for wild-type and mutant cardiac preparations, respectively. The PAF-mediated negative inotropism results inhibited by L-NAME in wild-type samples and restored by SNP in $PI3K\gamma^{-/-}$ atria. $**P < 0.01$.

3. Results

3.1. Protection from PAF-induced cardiac contractile depression in PI3K γ -null hearts

In response to ischemia, cardiac function is depressed by the release of autacoids like PAF, a potent inflammatory mediator [17] known to induce a negative inotropic effect [18,19]. PAF binds to a GPCR and activation of PI3K γ could be a possible downstream signaling event. To investigate whether PI3K γ played a role in mediating PAF-dependent effects on cardiac function, the contractile response to PAF stimulation was evaluated in electrically paced wild-type and PI3K γ -null cardiac muscles. In wild-type atria, PAF induced the expected negative inotropic effect, reducing contractility to 60% of its basal value (Fig. 1A and C). Pretreatment with the PAF receptor antagonist WEB 2170 inhibited in wild-type cardiac muscle preparations the PAF-dependent negative inotropism, thus demonstrating the specific involvement of the PAF receptor in this process (Fig. 1C). Strikingly, when PAF was administered to PI3K γ -null atria the reduction of contractility could not be detected (Fig. 1B and C). Identical results were obtained studying the effect of lower doses of PAF (0.1 μ M) on Langendorff-perfused hearts: wild-type samples showed a $54.3 \pm 6.1\%$ reduction of contractility ($n=6$), and PI3K γ -deficient hearts were not affected ($106.8 \pm 7.4\%$ of basal contractility, $n=4$; $P<0.01$, wild-type versus mutant). These data thus demonstrate that PI3K γ relays signals mediating the PAF-mediated negative inotropic response.

3.2. Defective PAF-induced NO production in hearts lacking PI3K γ

It has previously been suggested that PAF modulates cardiac function by triggering the production of nitric oxide (NO) [20]. Concomitant administration of PAF and of the NO synthesis inhibitor *N*(ω)-nitro-L-arginine methyl ester (L-NAME) completely prevented the negative inotropic effect. The use 1 mM of *N*(G)-methyl-L-arginine (L-NMMA), a NO synthase inhibitor without antimuscarinic activity, provided identical results: $91.0 \pm 5.0\%$ of contractility before PAF administration in wild-type atria treated with L-NMMA and PAF. Addition of 1 mM L-arginine to L-NMMA-treated cardiac samples restored the effect of PAF ($63.3 \pm 1.5\%$ of contractility before PAF). Conversely, administration of the NO donor sodium nitroprusside (SNP) induced a comparable reduction of contractility in wild-type and PI3K γ -null cardiac muscle (Fig. 2). In addition, treatment of wild-type atria with Triton X-100 to remove endocardial endothelium did not modify the negative inotropic effect exerted by PAF ($59.5 \pm 2\%$ of basal contractility, $n=6$). These results suggest that myocardial PI3K γ is necessary for PAF-induced NO release. To study if the effect of PAF on contractility were depending on adenosine or acetylcholine release, atria were pretreated with inhibitors of the adenosine A₁ (8-cyclopentyl-1,3-dipropylxanthine, DPCPX 10 μ M) and acetylcholine muscarinic (Atropine 5 μ M) receptors. Neither A₁ nor M receptors blockade modified the negative inotropic response to PAF ($59.0 \pm 10.0\%$ and $59.5 \pm 6.5\%$ of basal contractility, respectively; $n=4$) in wild-type cardiac preparations, thus

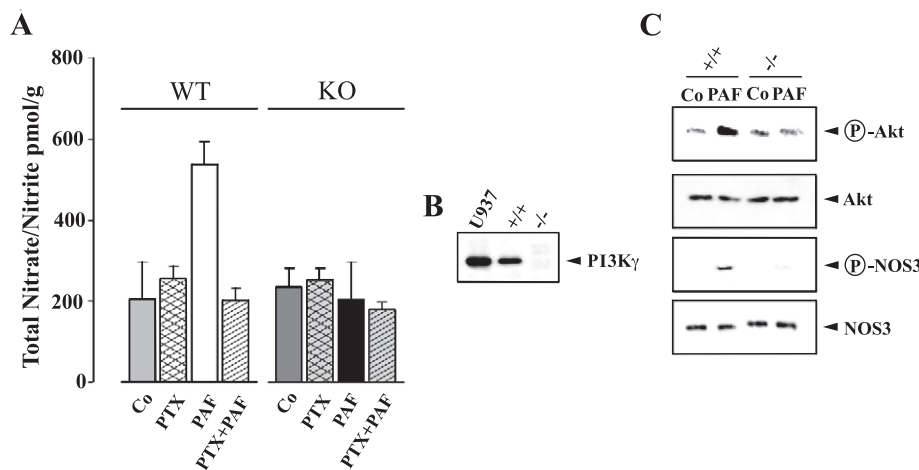


Fig. 3. Involvement of PI3K γ in the PAF-dependent NO production in hearts. (A) Measurement of the PAF-dependent total nitrite/nitrate production in wild-type (lighter bars) and PI3K γ ^{-/-} (darker bars) hearts. Co (gray bars; $n=4$ for each genotype), nitrate/nitrite concentration in resting condition; PTX (crossed bars; $n=3$ for each genotype), nitrate/nitrite concentration after treatment with pertussis toxin (PTX); PAF (white and black bars; $n=6$ for each genotype), nitrate/nitrite concentration after stimulation with 1 μ M PAF; PTX+PAF (hatched bars; $n=4$ for each genotype), nitrate/nitrite concentration in PAF-stimulated hearts pretreated with pertussis toxin. PAF-mediated NO production in the heart is blocked by PTX and by the lack of PI3K γ . (B) Immunoprecipitation analysis of PI3K γ expression in wild-type and PI3K γ -deficient murine hearts. Displayed are immunoprecipitates from a control cell line expressing PI3K γ (U937), and from wild-type (+/+) and PI3K γ ^{-/-} (-/-) hearts. (C) Detection of the phosphorylation state of PKB/Akt and NOS3 after PAF stimulation of wild-type (+/+) and PI3K γ ^{-/-} (-/-) hearts. Co, unstimulated control. PI3K γ is needed for PAF-dependent maximal phosphorylation of PKB/Akt and NOS3.

further indicating a crucial role for the PAF-dependent NO production.

Measurement of the total concentration of NO degradation products nitrite/nitrate in explanted wild-type hearts showed that PAF stimulation induces a threefold increase over the basal level of nitrite/nitrate concentration per gram of wet tissue (Fig. 3A). Since the pretreatment of mice with pertussis toxin blocks the PAF-mediated NO production, this process depends on a signaling pathway activating G_i (Fig. 3A). This effect was specific as treatment with PTX alone and in combination with PAF of wild-type and mutant cardiac tissue was equal to the untreated controls. Strikingly, the PAF-dependent NO production also requires PI3K γ activity because, in PI3K γ -null mice, PAF did not increase nitrite/nitrate concentration above background levels (Fig. 3A). To elucidate the mechanism underlying these effects we analyzed the phosphorylation of the endothelial NO synthase. Recent reports indicate that the protein kinase B (PKB)/Akt, which is phosphorylated and activated in a PI3K-dependent way, might phosphorylate and activate the NO synthase type 3 (NOS3) [21,22], an enzyme constitutively expressed by cardiomyocytes [23]. Consistently, PAF stimulation of hearts expressing PI3K γ (Fig. 3B) caused both PKB/Akt and NOS3 phosphorylation (Fig. 3C). By contrast, mutant hearts lacking PI3K γ (Fig. 3B) showed a marked reduction in PKB/Akt and NOS3 phosphorylation after PAF administration (Fig. 3C). These data thus indicate that PAF triggers NO production through a G_i -dependent pathway, involving the PI3K γ -dependent phosphorylation of PKB/Akt and NOS3.

3.3. Improved postischemic recovery in PI3K γ -null hearts

Because contractility is a critical parameter of cardiac function that seriously deteriorates after ischemia, we assessed the impact of the lack of PI3K γ in the postischemic recovery of hearts. To minimize indirect effects due to impaired inflammatory responses of PI3K γ -null mice [7–9], contractile force during ischemia/reperfusion was studied ex vivo in Langendorff-perfused hearts. Preischemic cardiac function of PI3K γ -null hearts was comparable to that of wild-type controls and during the period of global ischemia, ventricular developed force was equally depressed in both genotypes. However, after reperfusion, the recovery of contractile force, the rate of contractile force development and the rate of ventricular relaxation were significantly improved in PI3K γ -deficient hearts compared to wild-type controls ($P < 0.001$, $P = 0.02$, $P = 0.01$, respectively, by two-way ANOVA). At 20 min of reperfusion, wild-type hearts showed a maximal contractile recovery of 60%, while mutant hearts reached 80% of the preischemic value (Fig. 4A–C; $P < 0.05$, wild-type versus mutant hearts by Bonferroni post hoc test). This highlights a fundamental role of PI3K γ in depressing cardiac function in postischemic stress conditions.

To test whether this could be due to a decreased responsiveness to PAF, we analyzed the contractile recovery of

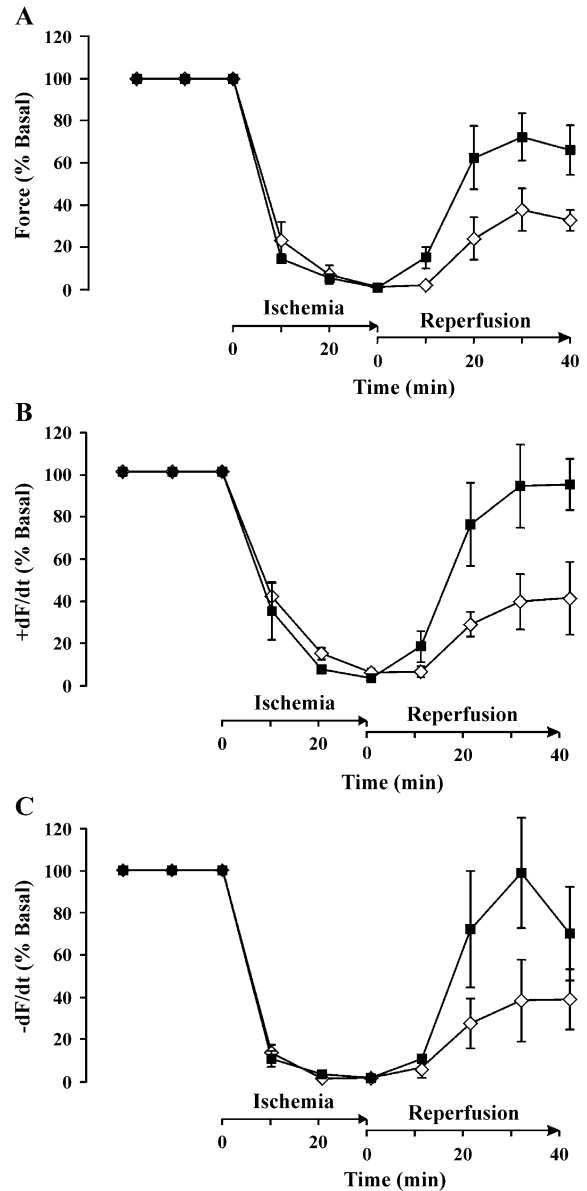


Fig. 4. Cardiac functions during ischemia and reperfusion in Langendorff-perfused PI3K γ -deficient and control hearts. Wild-type (white diamonds; $n=5$) and PI3K $\gamma^{-/-}$ (black squares; $n=5$) hearts were perfused in Langendorff mode with for 20 min and then subjected to 30 min of ischemia, followed by 40 min of reperfusion. (A) Left ventricular systolic contractile force as a percentage of baseline. (B) Rate of left ventricular maximal contractile force development over time ($+dF/dt$). (C) Rate of left ventricular maximal relaxation over time ($-dF/dt$). Baseline force of contraction was 1616.9 ± 123.1 and 1763.3 ± 156.4 mg for wild-type and mutant hearts, respectively. Maximal contraction velocity was 28.8 ± 2.3 and 31.4 ± 3.3 g/s for wild-type and mutant hearts, respectively. Maximal relaxation velocity was 23.2 ± 2.8 and 25.4 ± 3.5 g/s for wild-type and mutant hearts, respectively. Statistical difference of wild-type versus mutant: $P < 0.001$ as determined by two-way ANOVA.

wild-type and mutant mouse hearts subjected to ischemia/reperfusion after incubation with the PAF receptor antagonist WEB 2170. Interestingly, in wild-type cardiac muscles, this treatment caused a marked increase in postischemic contractility that was overlapping with that detected in PI3K γ -null

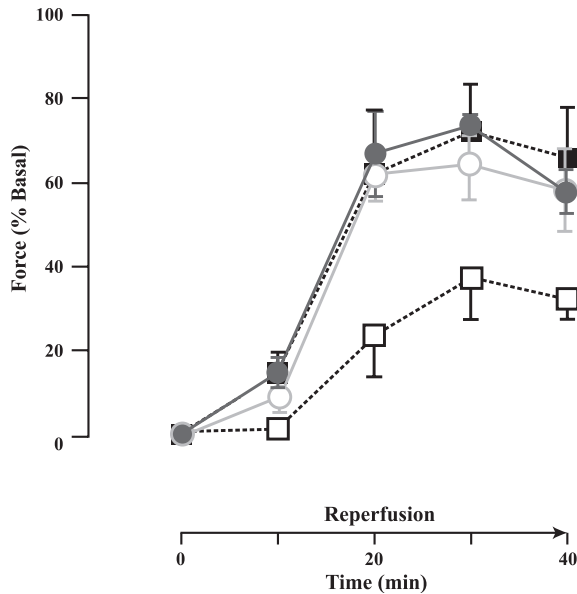


Fig. 5. Role of PAF in the alteration of cardiac contractility after ischemia/reperfusion. Wild-type (white squares/circles; $n=5$) and $PI3K\gamma^{-/-}$ (black squares/circles; $n=5$) hearts were perfused in Langendorff mode with for 20 min and then subjected to 30 min of ischemia, followed by 40 min of reperfusion. Hearts of both genotypes were incubated with (circles) or without (squares) the PAF receptor antagonists WEB 2170 (3 μ M) 10 min before ischemia. Left ventricular systolic contractile force is expressed as a percentage of baseline. Prior to reperfusion, contractile force was identical in all samples (1691.9 ± 137.3 and 1722 ± 141.8 mg for wild-type and mutant hearts, respectively). No significant statistical difference could be detected among untreated mutant hearts and WEB 2170-treated wild-type and mutant hearts.

hearts (Fig. 5). The fact that treatment of mutant hearts with WEB 2170 did not exert any further improvement in the contractile recovery (Fig. 5) suggests that the increased postischemic cardiac response of hearts lacking $PI3K\gamma$ was caused by the absence of response to PAF.

Experiments performed on isolated atria stimulated with PAF strongly suggested a link between the $PI3K\gamma$ -dependent nitric oxide production and the depression of cardiac contractility. To verify if $PI3K\gamma$ acts to cause postischemic injury through the activation of NO release, wild-type isolate hearts were subjected to ischemia/reperfusion in the presence of the NOS inhibitor L-NMMA (10 μ M). In agreement with the proposed mechanism, the blockade of NO synthesis in wild-type perfused hearts restored postischemic function ($75.0 \pm 7.2\%$ and $68.0 \pm 6.5\%$ of basal contractility at 20 and 30 min of reperfusion, respectively) to a similar extent of that detected in $PI3K\gamma$ -null isolated hearts.

4. Discussion

PAF is a potent biological mediator of inflammation that has been shown to be released in significant amounts during ischemia/reperfusion [24–26]. In normal conditions, PAF is present in minimal concentrations but can be released in

inflamed tissues by activated neutrophils, monocytes or endothelial cells [17]. The receptor for PAF belongs to the GPCR superfamily [17] and is present on cardiomyocytes where it relays signals causing a negative inotropic effect [27,28]. In the ischemic heart disease, PAF is considered as the major GPCR agonist depressing myocardial contractility and thus causing further distress to the ischemic organ [18,19,29].

Decreasing PAF production has proven to be effective in the protection from myocardial ischemia/reperfusion injury [30–32]. However, the identification of signaling events downstream PAF receptor activation might provide novel clues to develop more effective therapeutic intervention.

Here, we defined a specific role of $PI3K\gamma$ in the control of PAF-mediated reduction of cardiac contractility: hearts from mice lacking $PI3K\gamma$ appeared completely protected from the negative inotropic effect triggered by PAF. Interestingly, in wild-type hearts, the response to PAF was found mediated by the production of NO that required $PI3K\gamma$ for its PAF-mediated production. This finding is consistent with previous reports indicating that the release of NO could play a crucial role in the cardiac effects of PAF [20,33] and is further supported by our results indicating that L-NAME and L-NMMA completely blocks the negative inotropism induced by PAF. Our observations showing that stimulation of hearts lacking $PI3K\gamma$ with the NO donor SNP normally inhibits contractility further demonstrated that the signaling pathway triggered by NO is intact in mutant cardiac muscles and that $PI3K\gamma$ functions upstream NO production. The PAF receptor can be coupled to both G_q and G_i types of G proteins to exert different biological responses [34]. Our data showing that blocking G_i with pertussis toxin inhibits PAF-induced NO production demonstrates that, in the heart, this pathway exclusively involves the G_i -coupled PAF receptor. In conclusion, these results strongly suggest a novel PAF-induced signal transduction mechanism implicating a G_i -dependent activation of $PI3K\gamma$ and a consequent phosphorylation of PKB/Akt and NOS3.

In this study, we reported that mice lacking $PI3K\gamma$ show a better contractile recovery after a global ischemic insult. Using a PAF antagonist in wild-type murine hearts subjected to ischemia/reperfusion we found that, in the mouse, most of the depression of postischemic contractility is due to the action of this autacoid. Interestingly, we also found that the protection exerted by the PAF receptor antagonist in wild-type hearts is very similar to that detected in hearts lacking $PI3K\gamma$. This observation, together with the finding that the PAF receptor antagonist did not further increase the contractile recovery in mutant hearts, strongly suggests that the unresponsiveness to PAF, caused by the lack of $PI3K\gamma$, is the major cause of the improved postischemic contractility of mutant hearts. In addition, we provided evidence that most of the contractile depression after reperfusion is mediated by the release of NO, thus suggesting that the improved performance detected in mutant hearts involves the lack of activation of a signaling cascade that causes, through PAF release

and PI3K γ activation, increased NO production. Indeed, this is in agreement with previous reports indicating that blockade of NOS enzymes in the postischemic heart of rats causes an increased contractile recovery [35]. Our findings thus indicate that, in the present experimental setting, PAF and PI3K γ -mediated signal transduction play a fundamental role. Nevertheless, several other causes for postischemic functional depression have been reported so far, including free radicals, KATP channels and β -adrenergic signaling. Although our data tend to exclude the involvement of these factors, it cannot, however, be excluded that in an *in vivo* context, other pathophysiologic processes are more important in causing postischemic decrement of cardiac function. Consistently with this hypothesis, deletion of the NOS3 gene in mice causes contrasting results depending on the presence or on the absence of blood perfusion in the heart. Although isolated and Langendorff-perfused NOS3-deficient hearts show an increased contractile recovery after ischemia [36], temporary coronary artery occlusion *in vivo* causes exacerbation of the postischemic recovery and a larger infarct size [37]. Further studies will thus be required to assess the role of PI3K γ in an *in vivo* model of cardiac ischemia/reperfusion. Nevertheless, the data in the present report indicate that PI3K γ plays a crucial role in the contractile dysfunction following ischemia/reperfusion, possibly suggesting that modulation of PI3K γ activity might provide new valuable therapeutic strategies to improve heart performance after an ischemic insult.

Acknowledgements

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