Original Research

Signaling pathways involved in postconditioning-induced cardioprotection of human myocardium, *in vitro*

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Abstract

We examined the respective role and relationship between protein kinase C (PKC), mitochondrial adenosine triphosphate-sensitive potassium (mitoK$_{ATP}$) channel and p38 mitogen-activated protein kinase (MAPK) in postconditioning of human myocardium, *in vitro*. Isometrically contracting, isolated human right atrial trabeculae were exposed to 30 min hypoxia and 60 min reoxygenation. Phorbol 12-myristate 13-acetate (a PKC activator), diazoxide (a mitoK$_{ATP}$ opener) and anisomycin (a p38 MAPK activator) were superfused in early reoxygenation alone and with calphostin C (a PKC inhibitor), 5-hydroxydecanoate (5-HD, a mitoK$_{ATP}$ channel inhibitor) and SB 202190 (a p38 MAPK inhibitor). Developed force at the end of the 60 min reoxygenation (FoC$_{60}$) period was compared between groups (mean $\pm$ SD). Phorbol 12-myristate 13-acetate (91 $\pm$ 4% of baseline), diazoxide (85 $\pm$ 5% of baseline) and anisomycin (90 $\pm$ 4% of baseline) enhanced the FoC$_{60}$ as compared with the control group (53 $\pm$ 7% of baseline, $P < 0.0001$). The enhanced FoC$_{60}$ induced by phorbol 12-myristate 13-acetate was abolished by calphostin C (52 $\pm$ 5% of baseline) and 5-HD (56 $\pm$ 3% of baseline), but not by SB 202190 (90 $\pm$ 8%). The diazoxide-induced recovery of FoC$_{60}$ was attenuated by 5-HD (55 $\pm$ 6% of baseline), but was not modified by calphostin C (87 $\pm$ 5% of baseline) and SB 202190 (90 $\pm$ 8% of baseline). The anisomycin-induced recovery of FoC$_{60}$ was abolished by calphostin C (61 $\pm$ 9% of baseline) and SB 202190 (52 $\pm$ 8% of baseline), but not by 5-HD (88 $\pm$ 6% of baseline). In conclusion, PKC activation, opening of mitoK$_{ATP}$ channels and p38 MAPK activation in early reoxygenation induced the postconditioning of human myocardium, *in vitro*. Furthermore, PKC activation was upstream of the opening of mitoK$_{ATP}$ channels; p38 MAPK acted on PKC. Therefore, mitoK$_{ATP}$ and p38 MAPK seemed to be involved in two independent pathways.

Keywords: postconditioning, human myocardium, protein kinase C, mitochondrial adenosine triphosphate-sensitive potassium channels, p38 mitogen-activated protein kinase

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Introduction

Following myocardial ischemia, reperfusion is mandatory for the salvage of myocytes and thus cardiac function. However myocardial necrosis and apoptosis are enhanced through 'reperfusion injury', leading to further myocardial damage. Reperfusion injury may be reduced by cardioprotective strategies such as postconditioning (PostC), which provides a more clinically amenable approach to cardioprotection.[1] PostC, first described by Zhao *et al.*,[2] as brief episodes of ischemia immediately at the onset of reperfusion after a prolonged ischemic insult, has been shown to reduce infarct size.

Although PostC has several similarities with preconditioning (PreC), the precise cascade of cellular events involved in PostC remains under investigation. It is well recognized that protein kinase C (PKC) mediates the cardioprotection of ischemic PostC[3,4] or anesthetic PostC.[5] It has also been shown that opening of mitochondrial adenosine triphosphate-sensitive potassium (mitoK$_{ATP}$) channels mediated the cardioprotection induced by ischemic and

pharmacological PostC.[5–9] Finally, the cardioprotection resulting from ischemic PostC has been linked with the activation of several survival kinases such as the phosphatidylinositol-3-kinase, Akt and extracellular-regulated kinase 1/2 mitogen-activated protein kinase (MAPK).[10] However, there are controversies on the protective or deleterious effect of some kinases,[11,12] such as the p38 MAPK.[10] Several studies have suggested the involvement of p38 MAPK, in the cascade of cellular events triggered during ischemia–reperfusion, and also following hypoxic PostC[13] and anesthetic PostC.[5]

Hypoxic PreC has been shown to be triggered by p38 MAPK activation and mediated by PKC.[14] In contrast, in adenosine-induced PreC, PKC is activated, pointing to the p38 MAPK pathway as a potential distal effector.[15] Nevertheless, studies have shown that following ischemic PreC, PKC activation can modulate mitoK$_{ATP}$ channel activation.[16,17] Subsequently, opening of mitoK$_{ATP}$ channels could activate p38 MAPK by generation of radical oxygen species.[18,19] Importantly, the relationship between PKC, mitoK$_{ATP}$ channels and p38 MAPK in the PostC pathway remains unknown.

The controversial results reported about mechanisms involved in cardioprotection can be explained mainly by the differences between protocols applied to induce ischemia–reperfusion, and by the choice of animal species. Although one of the goals of experimental studies using animal models is translation to humans, species differences remain a critical issue.[20] Importantly, animals used to study the effect of ischemia–reperfusion had no coexisting diseases, no chronic treatment and are often young adults. The present experimental model and pharmacological approach may be considered as advantageous since it provides the unique opportunity to examine the mechanisms involved in cardioprotective strategies in human myocardium exposed to patients' chronic treatment and to anesthetic agents.

The aim of the present study was to examine the role and the relationship between PKC, mitoK$_{ATP}$ channels and p38 MAPK in PostC-induced cardioprotection and to compare them with experimental data using animal models.

Materials and methods

After the approval of the local medical ethics committee (Comité de Protection des Personnes Nord Ouest III, Caen, France) and written informed consent, right atrial appendages were obtained, during cannulation for cardiopulmonary bypass, from patients scheduled for routine coronary artery bypass surgery and aortic valve replacement. All patients received total intravenous anesthesia with propofol and remifentanil. Patients with chronic atrial arrhythmia and with diabetes mellitus treated with insulin or oral hypoglycemic agents were excluded from the study.[5]

Experimental conditions

Right atrial trabeculae (one per appendage) were dissected and suspended vertically between an isometric force transducer (MLT0202, ADInstruments, Sydney, Australia) and a stationary stainless clip in a 200 mL jacketed reservoir filled with daily prepared Tyrode's modified solution containing (mmol/L) 120 NaCl, 3.5 KCl, 1.1 $MgCl_2$, 1.8 NaH_2PO_4, 25.7 $NaHCO_3$, 2.0 $CaCl_2$ and 5.5 glucose. The jacketed reservoir was maintained at 34°C by a thermostatic water circulator (Polystat micropros, Bioblock, Illkirch, France). The bathing solution was insufflated with carbogen (95% O_2–5% CO_2), resulting in a pH of 7.40 and a partial pressure of oxygen of 600 mmHg. Isolated muscles were field-stimulated at 1 Hz by two platinum electrodes with rectangular wave pulses of 5 ms duration 20% above threshold (CMS 95107, Bionic Instrument, Paris, France).

Trabeculae were equilibrated for 60–90 min to allow stabilization of their optimal mechanical performance at the apex of the length-active isometric tension curve. The force developed was measured continuously, digitized at a sampling frequency of 400 Hz and stored on a Writable Compact Disc for analysis (MacLab, AD Instrument, Sydney, Australia).

At the end of the experiment, the muscle cross-sectional area was calculated from its weight and length, assuming a cylindrical shape and a density of 1. It has been previously shown that the atrial trabeculae contained 90% of myocytes that are organized in parallel bundles. To avoid core hypoxia and to allow stability of mechanical parameters throughout the experiments,[21] trabeculae included in the study must have a cross-sectional area $<1.0\ mm^2$, a force of contraction normalized per cross-sectional area $>5.0\ mN/mm^2$ and a ratio of resting force/total force <0.45.

Experimental protocol

At the end of the stabilization period, the trabeculae were randomized (sealed envelopes) to one of experimental groups. In all groups, hypoxia–reoxygenation was performed by replacing 95% O_2–5% CO_2 with 95% N_2–5% CO_2 in the buffer for 30 min, followed by a 60 min oxygenated recovery period. The experimental groups studied are depicted in Figure 1.

In the control group (Control; $n = 20$) trabeculae were exposed to hypoxia–reoxygenation alone (Figure 1).

The respective role of PKC, mitoK$_{ATP}$ channels and p38 MAPK in PostC signaling pathway was studied using an activator of PKC (phorbol 12-myristate 13-acetate; 1 μmol/L PMA), an opener of mitoK$_{ATP}$ channel (100 μmol/L diazoxide) and an activator of p38 MAPK (100 nmol/L anisomycin) administered during the first 15 min of reoxygenation (Figure 1).

In separate experimental groups ($n = 6$ in each group), each activator was administered alone, and in the presence of 1 μmol/L calphostin C, a PKC inhibitor; 800 μmol/L 5-hydroxy decanoate (5-HD), a mitoK$_{ATP}$ channel inhibitor; or 1 μmol/L SB 202190, a p38 MAPK inhibitor ($n = 6$ in each group). During these experiments, administration of calphostin C, 5-HD and SB 202190 started 5 min before and during the first 15 min of reoxygenation (Figure 1).

In addition, the effect of calphostin C, 5-HD and SB 202190 alone was studied in separate groups exposed to 1 μmol/L calphostin C ($n = 6$), 800 μmol/L 5-HD ($n = 6$)

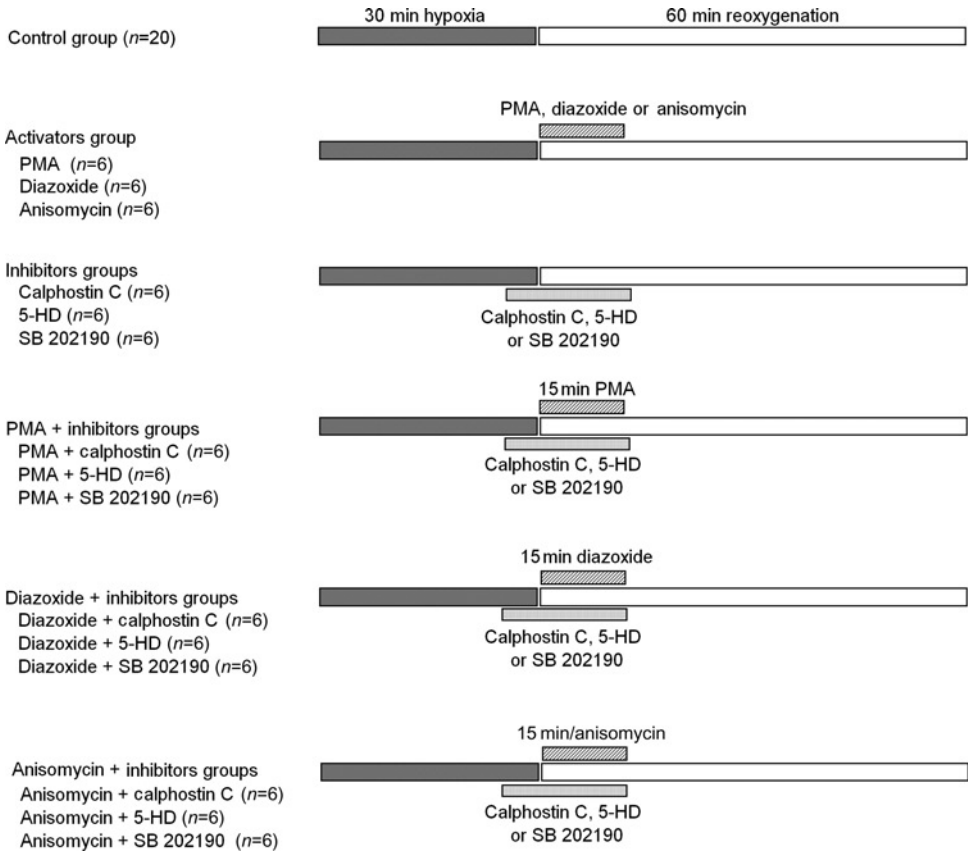

Figure 1 Schematic diagram depicting the experimental protocol. In PMA and PMA + inhibitor groups, PMA was administered at 1 μmol/L. In diazoxide and diazoxide + inhibitor groups, diazoxide was administered at 100 μmol/L. In anisomycin and anisomycin + inhibitor groups, anisomycin was administered at 100 nmol/L. In inhibitor groups, calphostin C was administered at 1 μmol/L, 5-HD was administered at 800 μmol/L and SB 202190 was administered at 1 μmol/L. 5-HD, 5-hydroxy-decanoate; PMA, phorbol 12-myristate 13-acetate

and 1 μmol/L SB 202190 ($n = 6$) 5 min before and during the first 15 min of reoxygenation.

Concentrations used have been validated in previous experimental studies in human myocardium, *in vitro* at concentrations that inhibited or activated cardioprotection.[5,22]

Chemicals

Calphostin C, diazoxide and 5-HD were purchased from Calbiochem VWR International (Fontenay sous Bois, France), and anisomycin, PMA and SB 202190 were obtained from Sigma Aldrich (Saint Quentin Fallavier, France).

Statistical analysis

The endpoint of the study was the recovery of FoC at 60 min of reoxygenation (FoC_{60}, expressed as percent of baseline). Based on the previous results obtained in our laboratory, power analysis calculated a group size of $n = 5$ to detect a difference of 40% in FoC (control and groups without PostC effect: $FoC_{60} = 50 \pm 9\%$ of baseline, and groups with a PostC effect: $FoC_{60} = 90 \pm 9\%$ of baseline) with a power of 0.8 at an alpha-level of 0.05. The number of experiments per group was calculated based on an one-way analysis of variance (ANOVA) with 13 groups. Data are expressed as mean $\pm$ SD. Baseline values of main mechanical parameters, age, preoperative left ventricular ejection fraction and FoC_{60} were compared by univariate analysis of variance with group factor as the independent variable. If the P value was <0.05, a Bonferroni *post hoc* analysis was performed. Within-group data were analyzed over time using analysis of variance for repeated measures and Bonferroni *post hoc* analysis with group factor and time (baseline, hypoxia 5, 10, 20 and 30 min, and reoxygenation 5, 10, 20, 30, 40, 50 and 60 min) as independent variables.

Results

The 110 right atrial trabeculae studied were obtained from patients whose demographic data, preoperative drug treatment, comorbidities and preoperative left ventricular ejection fraction are reported in Table 1. There were no significant differences in baseline values for L_{max}, cross-sectional area and force of contraction normalized per cross-sectional area between experimental groups (Table 2).

Effect of hypoxia–reoxygenation

In the control group, hypoxia induced a marked decrease in FoC ($14 \pm 11\%$ of baseline after 30 min of hypoxia) whereas reoxygenation resulted in a partial recovery of FoC_{60} ($51 \pm 9\%$ of baseline).

Table 1 Patients' demographic data, preoperative drug treatments, comorbidities and preoperative left ventricular ejection fraction

Groups and heart disease	Age (y)	Preoperative drug treatments	Comorbidities	LVEF (%)
Control AVR ($n = 9$); CABG ($n = 11$)	65 ± 9	ACE (8), ßAB (8), BZD (2), CA (2), COR (1), FUR (1), STA (10), TNT (1)	HLD (10), HTN (8), SMO (8)	63 ± 7
PMA AVR ($n = 1$); CABG ($n = 5$)	53 ± 12	ACE (5), ßAB (5), BZD (0), CA (0), COR (0), FUR (0), STA (5), TNT (0)	HLD (3), HTN (2), SMO (5)	53 ± 11
PMA + calphostin C AVR ($n = 3$); CABG ($n = 3$)	69 ± 10	ACE (1), ßAB (4), BZD (0), CA (2), COR (0), FUR (1), STA (3), TNT (0)	HLD (5), HTN (1), SMO (3)	60 ± 18
PMA + 5-HD AVR ($n = 3$); CABG ($n = 3$)	67 ± 19	ACE (2), ßAB (4), BZD (2), CA (0), COR (0), FUR (0), STA (4), TNT (2)	HLD (4), HTN (0), SMO (3)	71 ± 9
PMA + SB 202190 AVR ($n = 2$); CABG ($n = 4$)	64 ± 5	ACE (1), ßAB (4), BZD (1), CA (1), COR (0), FUR (1), STA (4), TNT (1)	HLD (2), HTN (3), SMO (2)	67 ± 1
Diazoxide AVR ($n = 1$); CABG ($n = 5$)	71 ± 9	ACE (3), ßAB (3), BZD (2), CA (0), COR (0), FUR (2), STA (6), TNT (0)	HLD (3), HTN (6), SMO (4)	62 ± 7
Diazoxide + calphostin C AVR ($n = 2$); CABG ($n = 4$)	62 ± 19	ACE (3), ßAB (6), BZD (2), CA (0), COR (0), FUR (1), STA (6), TNT (0)	HLD (3), HTN (4), SMO (0)	58 ± 6
Diazoxide + 5-HD AVR ($n = 3$); CABG ($n = 3$)	70 ± 10	ACE (1), ßAB (3), BZD (1), CA (2), COR (0), FUR (1), K^+A (2), MOL (0), STA (4), TNT (0)	HLD (4), HTN (2), SMO (2)	51 ± 15
Diazoxide + SB 202190 AVR ($n = 3$); CABG ($n = 3$)	68 ± 9	ACE (4), ßAB (3), BZD (0), CA (0), COR (0), FUR (2), STA (4), TNT (1)	HLD (5), HTN (2), SMO (2)	53 ± 19
Anisomycin AVR ($n = 3$); CABG ($n = 3$)	74 ± 10	ACE (6), ßAB (6), BZD (1), CA (2), COR (0), FUR (1), STA (4), TNT (0)	HLD (5), HTN (6), SMO (0)	78 ± 13
Anisomycin + calphostin C AVR ($n = 1$); CABG ($n = 5$)	65 ± 10	ACE (4), ßAB (3), BZD (2), CA (0), COR (0), FUR (2), STA (4), TNT (0)	HLD (5), HTN (4), SMO (1)	56 ± 17
Anisomycin + 5-HD AVR ($n = 4$); CABG ($n = 2$)	56 ± 5	ACE (2), ßAB (4), BZD (0), CA (2), COR (0), FUR (0), STA (2), TNT (0)	HLD (3), HTN (5), SMO (2)	62 ± 12
Anisomycin + SB AVR ($n = 5$); CABG ($n = 1$)	71 ± 7	ACE (4), ßAB (3), BZD (0), CA (1), COR (0), FUR (1), STA (4), TNT (0)	HLD (2), HTN (3), SMO (2)	68 ± 8
Calphostin C AVR ($n = 5$); CABG ($n = 1$)	68 ± 10	ACE (2), ßAB (2), BZD (1), CA (1), COR (0), FUR (0), STA (4), TNT (1)	HLD (3), HTN (0), SMO (0)	73 ± 6
5-HD AVR ($n = 4$); CABG ($n = 2$)	72 ± 5	ACE (3), ßAB (3), BZD (1), CA (0), COR (0), FUR (2), STA (4), TNT (0)	HLD (4), HTN (4), SMO (4)	69 ± 17
SB 202190 AVR ($n = 3$); CABG ($n = 3$)	68 ± 9	ACE (2), ßAB (3), BZD (3), CA (2) COR (0), FUR (0), STA (0), TNT (0)	HLD (4), HTN (4), SMO (0)	72 ± 5

AVR, aortic valve replacement; CABG, coronary artery bypass graft; ACE, angiotensin-converting enzyme inhibitors; ßAB, ß-adrenergic blocking drugs; BZD, benzodiazepine; CA, calcium channel antagonists; COR, amiodarone; FUR, furosemide; 5-HD, 5-Hydroxy-decanoate; LVEF, preoperative left ventricular ejection fraction; HLD, hyperlipidemia; HTN, hypertension; PMA, phorbol 12-myristate 13-acetate; SMO, smoking; STA, statins; TNT, nitroglycerin
The number in parentheses after heart disease and drug abbreviation indicate the number of patients. Age and LVEF are expressed as mean ± SD

Table 2 Control values of main mechanical parameters of human right atrial trabeculae

Experimental groups	L_{max} (mm)	CSA (mm^2)	AF ($mN\ mm^{-2}$)	RF/TF
Control ($n = 20$)	7.3 ± 1.7	0.59 ± 0.25	23 ± 10	0.36 ± 0.13
PMA ($n = 6$)	5.7 ± 1.2	0.46 ± 0.22	28 ± 12	0.30 ± 0.05
PMA + calphostin C ($n = 6$)	5.8 ± 1.2	0.47 ± 0.15	29 ± 13	0.32 ± 0.09
PMA + 5-HD ($n = 6$)	6.2 ± 1.2	0.52 ± 0.20	29 ± 13	0.24 ± 0.10
PMA + SB 202190 ($n = 6$)	6.3 ± 1.8	0.55 ± 0.21	25 ± 15	0.25 ± 0.10
Diazoxide ($n = 6$)	7.0 ± 1.8	0.48 ± 0.20	27 ± 14	0.29 ± 0.12
Diazoxide + calphostin C($n = 6$)	6.4 ± 1.4	0.57 ± 0.19	29 ± 15	0.22 ± 0.07
Diaz + 5-HD ($n = 6$)	4.9 ± 1.0	0.46 ± 0.21	25 ± 6	0.31 ± 0,08
Diaz + SB 202190 ($n = 6$)	7.2 ± 2.3	0.54 ± 0.22	28 ± 12	0.27 ± 0.08
Anisomycin ($n = 6$)	6.2 ± 1.5	0.47 ± 0.15	27 ± 11	0.24 ± 0.05
Anisomycin + calphostin C ($n = 6$)	6.8 ± 1.2	0.39 ± 0.17	27 ± 13	0.36 ± 0.13
Anisomycin + 5-HD ($n = 6$)	5.7 ± 1.5	0.51 ± 0.14	26 ± 11	0.29 ± 0.10
Anisomycin + SB 202190 ($n = 6$)	7.0 ± 1.3	0.43 ± 0.13	28 ± 14	0.30 ± 0.13
Calphostin C ($n = 6$)	5.3 ± 1.0	0.47 ± 0. 18	21 ± 7	0.33 ± 0.10
5-HD ($n = 6$)	5.9 ± 0.9	0.43 ± 0.09	25 ± 9	0.32 ± 0.07
SB ($n = 6$)	7.2 ± 1.9	0.48 ± 0.20	25 ± 5	0.32 ± 0.10

Data are mean ± SD
AF, acting isometric force normalized per cross-sectional area; Aniso, anisomycin; Cal, calphostin C; CSA, cross-sectional area; 5-HD, 5-hydroxy-decanoate; L_{max}, maximal length at the apex of the length-active force curve; PMA, phorbol 12-myristate 13-acetate; RF/TF, ratio of resting force on total force

Effect of phorbol 12-myristate 13-acetate, diazoxide and anisomycin on hypoxia–reoxygenation

As compared with the control group, PMA (91 ± 4% of baseline; $P < 0.0001$), diazoxide (85 ± 5% of baseline; $P < 0.0001$) and anisomycin (90 ± 4% of baseline; $P < 0.0001$) increased the FoC_{60}. There was no difference in the FoC_{60} measured in PMA, diazoxide and anisomycin groups ($P > 0.05$; Figure 2).

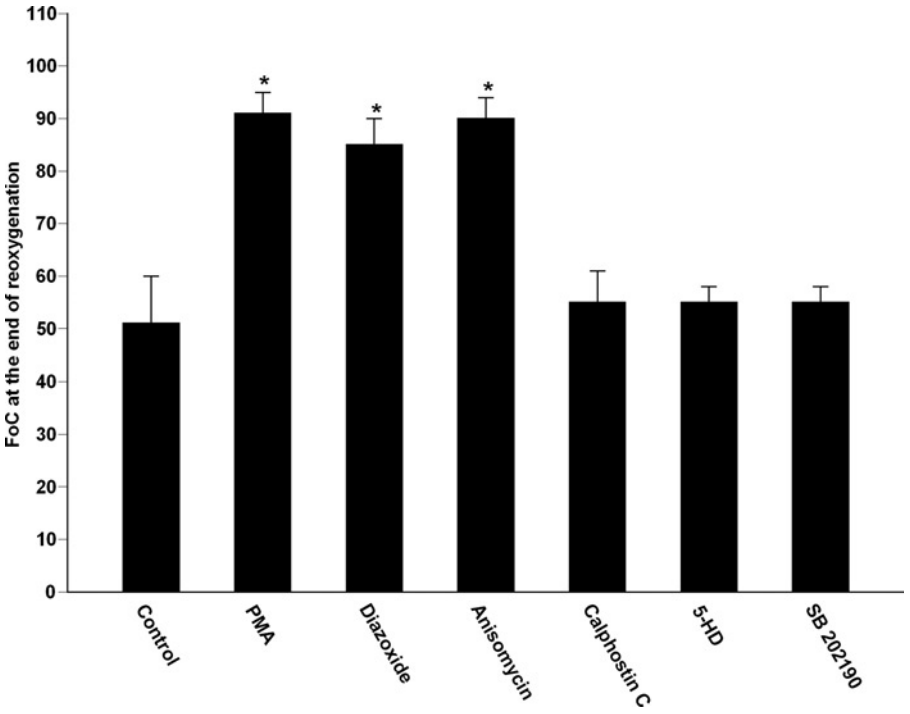

Figure 2 Effect of administration of PMA, diazoxide, anisomycin, calphostin C, 5-HD and SB 202190 at the beginning of reoxygenation on the time course of force of contraction (FoC, expressed as percent of baseline) of isolated human right atrial trabeculae during a 30 min hypoxic challenge followed by a 60 min reoxygenation period. Control ($n = 20$), PMA ($n = 6$), diazoxide ($n = 6$), anisomycin ($n = 6$), calphostin C ($n = 6$), 5-HD ($n = 6$), SB 202190 ($n = 6$) groups. Data are presented as mean $\pm$ SD. $^{*}P < 0.0001$ versus control, calphostin C, 5-HD, SB 202190 groups. 5-HD, 5-hydroxy-decanoate; PMA, phorbol 12-myristate 13-acetate

Effect of 5-HD calphostin C and SB 202190

As compared with the control group ($51 \pm 9\%$ of baseline), calphostin C ($55 \pm 6\%$ of baseline; $P = 0.20$), 5-HD ($55 \pm 3\%$ of baseline; $P = 0.22$) and SB 202190 ($55 \pm 3\%$ of baseline; $P = 0.26$) did not significantly modify FoC_{60} (Figure 2).

Effect of calphostin C, 5-HD and SB 202190 on phorbol 12-myristate 13-acetate treatment

The enhanced recovery of FoC_{60} following PMA administration ($91 \pm 4\%$ of baseline) was abolished in the presence of calphostin C (PMA + calphostin C: $52 \pm 5\%$ of baseline, $P < 0.0001$ versus the PMA group) and 5-HD (PMA + 5-HD: $56 \pm 3\%$ of baseline, $P < 0.0001$ versus the PMA group), and was not modified in the presence of SB 202190 (PMA + SB 202190: $90 \pm 8\%$ of baseline; $P = 0.72$ versus the PMA group) (Figure 3).

Effect of calphostin C, 5-HD and SB 202190 on diazoxide treatment

The enhanced recovery of FoC_{60} following diazoxide administration ($85 \pm 5\%$ of baseline) was abolished in the presence of 5-HD (diazoxide + 5-HD: $55 \pm 6\%$ of baseline, $P < 0.0001$ versus the diazoxide group), but was not modified neither in the presence of calphostin C (diazoxide + calphostin C: $87 \pm 5\%$ of baseline, $P = 0.58$ versus the diazoxide group) nor in the presence of SB 202190 (diazoxide + SB 202190: $90 \pm 8\%$ of baseline, $P = 0.14$ versus the diazoxide group) (Figure 3).

Effect of calphostin C, 5-HD and SB 202190 on anisomycin treatment

The enhanced recovery of FoC_{60} following anisomycin administration ($90 \pm 4\%$ of baseline) was abolished in the presence of SB 202190 (anisomycin + SB 202190: $52 \pm 8\%$ of baseline, $P < 0.0001$ versus the anisomycin group) and calphostin C (anisomycin + calphostin C: $61 \pm 9\%$ of baseline, $P < 0.0001$ versus the anisomycin group), but not in the presence of 5-HD (anisomycin + 5-HD: $88 \pm 6\%$ of baseline, $P = 0.62$ versus the anisomycin group) (Figure 3).

Discussion

The major findings of the current study are as follows: PKC activation, opening of mitoK$_{ATP}$ channel and p38 MAPK activation in early reoxygenation enhanced the recovery of contractile force. Furthermore, PKC activation is upstream of mitoK$_{ATP}$ channel opening. There was a link between p38 MAPK and PKC. In addition, mitoK$_{ATP}$ channel and p38 MAPK seemed to be implicated in two independent pathways in the cascade of PostC.

First, our results demonstrated that PKC activation by PMA administration in early reoxygenation enhanced the recovery of FoC following hypoxia–reoxygenation as compared with the control group. This is in agreement with previous studies in mammalian species: PMA administered at the onset of reperfusion could protect ischemic rabbit hearts, *in vitro*,[23] and limited the infarct size.[3] Moreover, our data showed that PMA-induced PostC is dependent on PKC activation, since cardioprotective effects of PMA

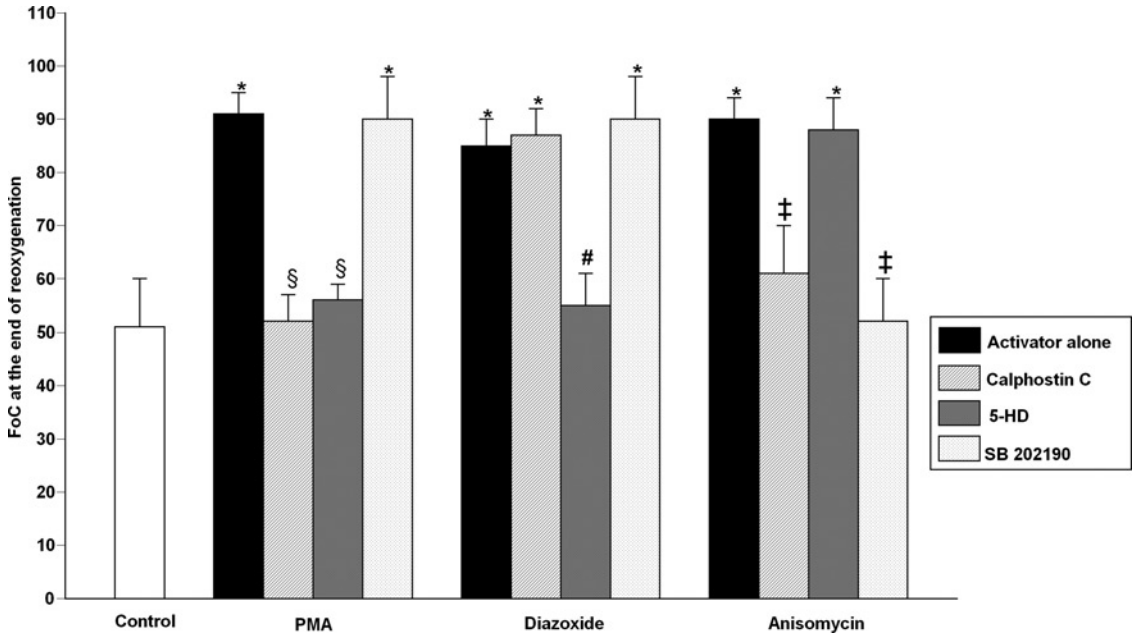

Figure 3 Recovery of force of contraction of isolated human right atrial trabeculae at the end of the 60 min reoxygenation period (FoC_{60}) after the 30 min hypoxic challenge in groups exposed to phorbol 12-myristate 13-acetate (PMA; $n = 6$) alone, and in the presence of calphostin C ($n = 6$), 5-HD ($n = 6$) and SB202190 ($n = 6$); diazoxide ($n = 6$) alone and in the presence of calphostin C ($n = 6$), 5-HD ($n = 6$), SB202190 ($n = 6$); anisomycin ($n = 6$) alone and in the presence of calphostin C ($n = 6$), 5-HD ($n = 6$) and SB 202190 ($n = 6$). Data are mean $\pm$ SD. $^{*}P < 0.0001$ versus the control group, $^{\S}P < 0.0001$ versus the PMA group, $^{\#}P < 0.0001$ versus the diazoxide group, $^{\ddagger}P < 0.0001$ versus the anisomycin group. 5-HD, 5-hydroxy-decanoate; PMA, phorbol 12-myristate 13-acetate

were abolished by pretreteament with calphostin C. In agreement with the data published by Philipp *et al.*,[3] PMA-induced PostC was aborted by co-administration of another PKC antagonist, chelerythrine, in rabbit *in vivo*.

Our results also showed that diazoxide, commonly used as an opener of the $mitoK_{ATP}$ channels, induced PostC in human myocardium *in vitro*. The present data confirm and extend the previous demonstration, showing that diazoxide exerts a protective effect against ischemia/reperfusion injury, when administered at the onset of reperfusion in isolated rat hearts.[24] However, these authors showed that it was necessary to administer diazoxide in an intermittent manner during early reperfusion to trigger PostC, whereas a continuous administration was not cardioprotective.[24] These divergent data can be explained by the difference between the different protocols for ischemia–reperfusion, the choice of the species (rat versus human) and the endpoint of the study (infarct size versus contractility). In addition, we showed that diazoxide–induced PostC was abolished by 5-HD, suggesting that cardioprotection by diazoxide was dependent on the opening of $mitoK_{ATP}$ channels. Recent data demonstrated the importance of $mitoK_{ATP}$ in mediating PostC, in dog heart *in vivo*.[7] Additionally 5-HD blocked the protection of PostC, implying a role for $mitoK_{ATP}$ channels in PostC, in isolated rabbit and rat hearts.[6,24] Nevertheless, the specificity of 5-HD has recently been questioned since metabolic effects independent of $mitoK_{ATP}$ channels (i.e. inhibition of respiratory chain complexes) have been shown.[25]

At the present time, it remains controversial whether activation of p38 MAPK is beneficial or deleterious in ischemic–reperfused myocardium.[10,13–15,18] Studies have suggested that p38 MAPK activation was required during myocardial PreC[22,26] and PostC[5] signaling cascade in human myocardium *in vitro*, whereas others have shown that its inactivation mediated cardioprotection following ischemic PostC and PreC.[13,15]

Our results show that anisomycin, a p38 MAPK activator, administered during the first minute of reoxygenation postconditioned human myocardium, *in vitro*, and this effect was similar to that observed in the presence of PMA or diazoxide. To our best knowledge, there is only one other study investigating the involvement of p38 MAPK in PostC, which arrived at almost the opposite conclusions.[13] This study showed that activation of p38 MAPK by anisomycin abrogated the cardioprotective effect of hypoxic PostC, in cultured rat cardiomyocytes subjected to prolonged hypoxia/reoxygenation.[13] Moreover, the inhibition of p38 MAPK induced cardiprotective effects.[13] Interestingly, we have shown that administration of SB 202190, a p38 MAPK inhibitor, alone did not modify the FoC_{60} in comparison with the control group. The explanation of this discrepancy (between data in isolated human trabeculae and in rat cardiomyocytes) would be methodological differences in the experimental design, including the endpoints examined (apoptosis versus recovery of force of contraction), and especially species differences. The isolated trabeculae also have distinct advantages over more reductionistic models such as isolated cardiomyocytes.[27] With the preservation of functional gap junctions and presence of other cell types such as fibroblasts and endothelial cells, trabeculae are the smallest units of functional myocardium that reliably reciprocate the *in vivo* situation. Additionally, this may be because both the PreC-and-PostC induced cardioprotection of human myocardium was always abolished by p38 MAPK inhibitor administration (SB 202190 or SB

203580).[5,22,26] In contrast, studies with animal models have reported contradictory results on the involvement of p38 MAPK in cardioprotection.[13,15]

The relationship between PKC activation, the opening of mitoK$_{ATP}$ channels and the activation of p38 MAPK in the mechanism involved in the PostC-induced cardioprotection remains unclear. In the present study, we investigated the respective role of PKC, mitoK$_{ATP}$ channels and p38 MAPK in PostC signaling pathway in human myocardium *in vitro*.

Thus, the PMA-induced PostC was abolished in the presence of 5-HD, but the diazoxide-induced PostC was preserved in the presence of calphostin C (Figure 3). The present study also suggests that PKC activation is upstream of the opening of mitoK$_{ATP}$ channels in the cascade of cellular events leading to the cardioprotective effect of PostC in human myocardium. Previous data have suggested that PKC activation is upstream of mitoK$_{ATP}$ channel opening,[28,29] that PKC activation can modulate the activation of mitoK$_{ATP}$ channels,[30] and that activation of PKC-ϵ leads to phosphorylation and opening of mitoK$_{ATP}$.[16,17] Taken together, the present results suggest a signaling pathway in early reoxygenation by which mitoK$_{ATP}$ channel opening mediates PostC through PKC activation. Other studies are not consistent with the present results; thus, PKC has been shown to be translocated and/or activated in diazoxide-treated hearts and cardiomyocytes, suggesting that mitoK$_{ATP}$ channel opening was upstream of PKC activation.[31–34] These discrepancies may result from differences in experimental models, differences in study endpoints (i.e. myocardial function, infarct size, cell survival, protein expression, apoptosis), differences in the timing and duration of administration of activators and inhibitors, and the specificity of the factors tested.

Little information is available about the signaling cascade involving MAPKs in PKC-mediated cardioprotection. PKC-ϵ protects against ischemia, by activation of extracellular signal-regulated kinase and c-Jun N-terminal kinase members of the MAPK family in rabbits and rats both *in vivo* and *in vitro*.[35–37] In addition, it has been shown that PKC-ϵ may form a mitochondrial localized signaling complex with MAPKs.[38] Previous work has shown that p38 MAPK is downstream of PKC in hypoxic PreC.[17] In support of this finding, we showed that the cardioprotective effects of p38 MAPK activation (by anisomycin) at the onset of reoxygenation were blocked by PKC inhibition, while inhibition of PKC activation (by PMA) were not affected by p38 MAPK inhibition. This strongly suggests that PKC activation is downstream of p38 MAPK activation in the signaling cascade of PostC in human myocardium.

On the other hand, our data demonstrated that cardioprotection induced by opening of mito K$_{ATP}$ channels was not altered by p38 MAPK inhibition. Furthermore, the enhanced recovery of FoC$_{60}$, resulting from p38 MAPK activation, was observed in the presence of 5-HD, suggesting that mitoK$_{ATP}$ channel opening and p38 MAPK activation in early reoxygenation exerted their cardioprotective effects through independent signaling pathways. In contrast to these results, it has been previously shown that cardioprotection by the MAPK activator anisomycin was blocked by a mitoK$_{ATP}$ channel inhibitor, and the opening of mitoK$_{ATP}$ channels was shown to activate p38 MAPK[39]. In addition, opening of mitoK$_{ATP}$ channels leads to extracellular signal-regulated kinase (another MAPK) activation via the generation of mitochondria-derived radical oxygen species.[40]

Recent clinical studies suggested the beneficial effects of myocardial ischemic PostC following coronary angioplasty in acute myocardial infarction,[1] and cardiac valve surgery.[41] Importantly, ischemic PostC has recently been shown to provide short- and long-term myocardial beneficial effects in patients scheduled for primary angioplasty and stenting of acute myocardial infarction.[41] The ability to reproduce the cardioprotective effects of PostC with pharmacological agents raises the possibility that a drug may ultimately be introduced into clinical practice to treat human hearts undergoing ischemia/reperfusion.[42] In particular, for development of cardioprotective strategy specific to the population with advanced age, it was previously demonstrated in aged rat that δ-opioid-mediated cardioprotection was lost and involves failure to activate p38MAPK; direct activation of p38 MAPK by anisomycin restores the protection.[43]

Several limitations must be considered in the interpretation of the present results. First, the effects of anesthetic drugs, physiological conditions, including other diseases, or medical treatments received by the patients before obtaining the atrial appendages cannot be ruled out. Thus, it is of interest to examine the effect of cardioprotective strategies in conditions closely resembling the clinical situations. Second, although our experimental groups showed comparable demographic data (Table 1), age has been shown to impair the protective effects of ischemic PostC in senescent mouse hearts.[44–46] Importantly, our investigation included an important control group ($n =$ 20) that was equally affected by these potentially modifying factors. Third, our experiments were performed under moderate hypothermia (34°C) to ensure stability of trabeculae over time. It has been shown that hypothermia may decrease the sensitivity of mitoK$_{ATP}$ channels.[47] However, during surgical procedures moderate hypothermia may occur. Fourth, the specificity of activators and inhibitors used in the present study should be considered. Although SB 202190 and SB 203580 are widely used as p38 MAPK inhibitors, it has been suggested that they may also inhibit c-Jun NH_2-terminal kinase/stress-activated protein kinase at high concentrations. However, a concentration of 1 μmol/Lol/L has been shown to be specific for the inhibition of p38 MAPK.[48] Additionally, the p38 MAPK inhibitor SB 202190 has been shown to have a similar specificity to that of SB 203580.[49] Similarly, although anisomycin is a strong activator of p38 MAPK, it has also been shown to activate c-Jun NH_2-terminal kinase. Diazoxide and 5-HD have also been shown to modify mitochondrial respiratory chain complex.[25] Diazoxide is a relatively selective opener of the mitoK$_{ATP}$ channel, but it can also have nonspecific metabolic effects on mitochondrial function, in particular inhibition of succinate dehydrogenase.[50] Lim *et al.*[51] showed that diazoxide inhibited state 3 succinate

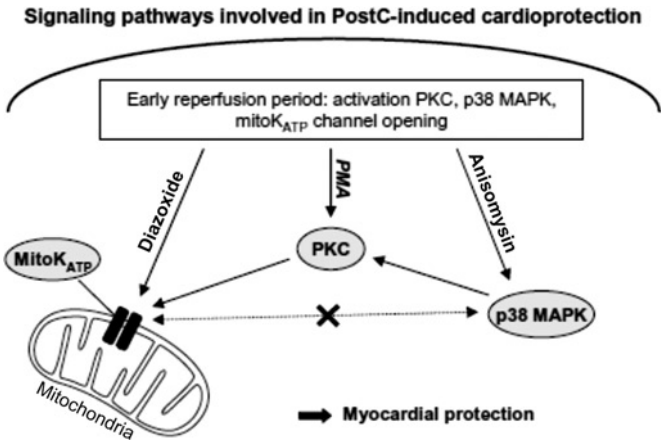

Figure 4 Proposed schematic representation of the signaling pathways leading to cardioprotection by PostC of the human myocardium, *in vitro*. In early reoxygenation, activation of PKC (via PMA administration), p38 MAPK (via anisomycin administration) and the opening of mitoK$_{ATP}$ channels (via diazoxide administration) induced myocardial PostC. Opening of mitoK$_{ATP}$ channels appears to be downstream of PKC activation; PKC activation is downstream of p38 MAPK activation. The mitoK$_{ATP}$ channels and p38 MAPK exert their cardioprotective effects independently. mitoK$_{ATP}$, mitochondrial adenosine triphosphate-sensitive potassium channel; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PostC, postconditioning; p38 MAPK, p38 mitogen-activated protein kinase

oxidation. Another limitation of the model is that it provides only indirect evidence of activation or inhibition of PKC and p38 MAPK; we are unable to perform Western blots or activity assays on human atrial trabeculae as the amount of tissue is quite small. Importantly, each inhibitor used in the present study has also been studied in the presence of the specific activator of its target. Thus the cardioprotection triggered by PMA at reoxygenation was abolished in the presence of calphostin C, that of diazoxide was abolished in the presence of 5-HD, and that of anisomycin was abolished by SB 202190 (Figure 3). Fifth, we studied isolated contracting human atrial trabeculae but not myocardial ventricular infarct size. Important functional and structural differences exist between atrial and ventricular myocardium. However, for ethical reasons it is not possible to obtain systematic ventricular biopsy in patients undergoing coronary artery bypass graft surgery or aortic valve replacement. Additionally, as described in myocardial PreC, the beneficial effects of PostC have also been described in reperfusion-induced arrhythmias[52] and myocardial conctractility.[53]

In conclusion, the current study shows that, in human myocardium PKC activation, the opening of mitoK$_{ATP}$ channels and the activation of p38 MAPK in early reoxygenation are induced by PostC of human myocardium. Opening of mitoK$_{ATP}$ channels appears to be downstream of PKC activation, which in turn appears to be downstream of MAPK activation. Nevertheless, mitoK$_{ATP}$ channels and p38 MAPK exert their cardioprotective effects independently (Figure 4). A better understanding of the mechanisms by which PKC, mitoK$_{ATP}$ and p38 MAPK are involved in signaling pathways that lead to cardioprotection after hypoxia will help devise novel therapeutic strategies.

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