Cardiovascular Research (2010) **88**, 324–333
doi:10.1093/cvr/cvq190

Consecutive pharmacological activation of PKA and PKC mimics the potent cardioprotection of temperature preconditioning

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Received 18 February 2010; revised 14 May 2010; accepted 8 June 2010; online publish-ahead-of-print 16 June 2010

Time for primary review: 25 days

Aims Temperature preconditioning (TP) provides very powerful protection against ischaemia/reperfusion. Understanding the signalling pathways involved may enable the development of effective pharmacological cardioprotection. We investigated the interrelationship between activation of protein kinase A (PKA) and protein kinase C (PKC) in the signalling mechanisms of TP and developed a potent pharmacological intervention based on this mechanism.

Methods and results Isolated rat hearts were subjected to TP, 30 min global ischaemia, and 60 min reperfusion. Other control and TP hearts were perfused with either sotalol (β-adrenergic blocker) or H-89 (PKA inhibitor). Some hearts were pre-treated with either isoproterenol (β-adrenergic agonist) or adenosine (PKC activator) that were given alone, simultaneously, or sequentially. Pre-treatment with isoproterenol, adenosine, and the consecutive isoproterenol/adenosine treatment was also combined with the PKC inhibitor chelerythrine. Cardioprotection was evaluated by haemodynamic function recovery, lactate dehydrogenase release, measurement of mitochondrial permeability transition pore opening, and protein carbonylation during reperfusion. Cyclic AMP and PKA activity were increased in TP hearts. H-89 and sotalol blocked the cardioprotective effect of TP and TP-induced PKC activation. Isoproterenol, adenosine, and the consecutive treatment increased PKC activity during pre-ischaemia. Isoproterenol significantly reduced myocardial glycogen content. Isoproterenol and adenosine, alone or simultaneously, protected hearts but the consecutive treatment gave the highest protection. Cardioprotective effects of adenosine were completely blocked by chelerythrine but those of the consecutive treatment only attenuated.

Conclusion The signal transduction pathway of TP involves PKA activation that precedes PKC activation. Pharmacologically induced consecutive PKA/PKC activation mimics TP and induces extremely potent cardioprotection.

Keywords Ischaemia • Mitochondria • Preconditioning • Protein kinase A • Protein kinase C

1. Introduction

Reperfusion following a prolonged period of ischaemia induces myocardial dysfunction and necrotic damage.[1] We have recently described a novel cardioprotective protocol in which hearts are subject to a few brief, transient hypothermic (26°C) episodes interspersed with normothermic perfusion prior to index ischaemia. Such temperature preconditioning (TP) is as good, if not better than ischaemic preconditioning (IP) in restoring haemodynamic function and reducing arrhythmias, oxidative stress, and lactate dehydrogenase (LDH) release.[2] We showed that TP involves a modest increase in reactive oxygen species (ROS) that activates protein kinase Cε (PKCε), although AMP-activated protein kinase may play some role. The cardioprotective effects of TP, like IP,[3] were associated with decreased oxidative stress at the end of ischaemia and during reperfusion. We proposed that this prevents opening of the mitochondria permeability transition pore (MPTP) leading to both improved contractile function and decreased necrotic damage.[2]

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The switch from hypothermic to normothermic perfusion during the TP protocol is accompanied by a rapid augmentation of haemodynamic function that subsequently returns to the initial value.[2] These changes could reflect β-adrenergic stimulation following a TP cycle with activation of cyclic AMP (cAMP)-dependent protein kinase A (PKA) that others have shown to be cardioprotective.[4] In this paper, we test this hypothesis and also study the temporal relationship between PKA and PKC activation in TP hearts. We demonstrate that PKA stimulation prior to PKC stimulation provides optimal cardioprotection. Understanding the signalling pathways and molecular targets through which TP exerts its effects may lead to the development of more effective pharmacological interventions.[5]

2. Methods

Unless otherwise stated, all biochemicals were from Sigma and general chemicals from Fischer Scientific or VWR-Jencons.

2.1 Experimental procedures

2.1.1 Heart perfusion and analysis of haemodynamic function

All procedures conform to the UK Animals (Scientific Procedures) Act 1986 and 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). Ethical approval was granted by the University of Bristol, UK (Investigator number ub/09/012). Male Wistar rats (250–260 g) were killed by stunning and cervical dislocation. Hearts (~0.75 g) were rapidly removed into ice-cold Krebs–Henseleit buffer (KH) and perfused in Langendorff mode with haemodynamic measurements of left ventricular developed pressure (LVDP), LV systolic pressure, LV end-diastolic pressure, work index (RPP), heart rate (HR), and time derivatives of pressure during contraction (+d*P*/d*t*) and relaxation (−d*P*/d*t*) as described previously[6] and detailed in Supplementary Methods.

2.1.2 Experimental groups

Four series of experiments were performed as shown schematically in *Figure 1*. Further details are provided in Supplementary Methods. In brief, after pre-ischaemia, global normothermic ischaemia (37°C) was induced for 30 min and then normothermic perfusion reinstated for 60 min. In Series 1, hearts were divided into two groups: control (15 hearts) and TP (12 hearts). TP hearts experienced three cycles of 2 min hypothermic perfusion at 26°C interspersed with 6 min normothermic perfusion prior to ischaemia. Samples of perfusate were collected for determination of LDH activity. Eight and six additional hearts of each group were freeze-clamped following 44 min pre-ischaemia and 15 min reperfusion, respectively, ground under liquid nitrogen, and stored at −80°C for later analysis of cAMP, PKA activity, and Akt and GSK3 phosphorylation.

In Series 2 (*Figure 1B*), six groups of six to eight hearts were employed; three control groups (C, CS, and CH) and three TP groups (TP, TPS, and TPH) in the presence or absence of 10 μM of the non-selective β-adrenergic blocker sotalol (CS and TPS) or 10 μM H-89 (PKA inhibitor) (CH and TPH). Sotalol and H-89 were washed out for 5 min before index ischaemia. Eight additional hearts of C, CH, TP, and TPH groups were freeze-clamped following 51 min pre-ischaemia for further analysis of PKC activity.

In Series 3 (*Figure 1C*), hearts were perfused with either adenosine, a PKC activator well known for its cardioprotective effects,[7] or isoproterenol, a non-selective β-adrenergic agonist widely used on isolated perfused heart. Hearts were divided into four groups (7–9 hearts in each): control, hearts perfused with 0.2 μM isoproterenol for 2 min followed by 10 min washout, hearts perfused with 30 μM adenosine for 5 min followed by 5 min washout, and hearts perfused with isoproterenol followed by perfusion with adenosine and 5 min washout. Eight additional non-ischaemic hearts of each group were freeze-clamped following 27 min KH perfusion (control group) or immediately after isoproterenol or adenosine treatment for later analysis of PKC activity. Eight more frozen pre-ischaemic hearts were used for measuring myocardial glycogen content in each group, whereas another 7–10 hearts of each group were used to prepare mitochondria after 30 min global ischaemia for the measurement of MPTP opening and analysis of protein carbonylation.

In Series 4 (*Figure 1C*), hearts were divided into eight groups (5–8 heats each): Group 1 control; Groups 2–4, hearts subjected to either isoproterenol, adenosine, or consecutive isoproterenol and adenosine treatment (see Series 3); in Groups 5–7, the PKC inhibitor chelerythrine (10 μM) was added 5 min before isoproterenol (Groups 5 and 7) or adenosine (Group 6) perfusion and removed prior to ischaemia. Chelerythrine at this concentration has no effect on heart recovery during reperfusion.[2] Hearts of Group 8 were perfused with 30 μM adenosine for 5 min with 0.2 μM isoproterenol also added after 1.5 min for 2 min.

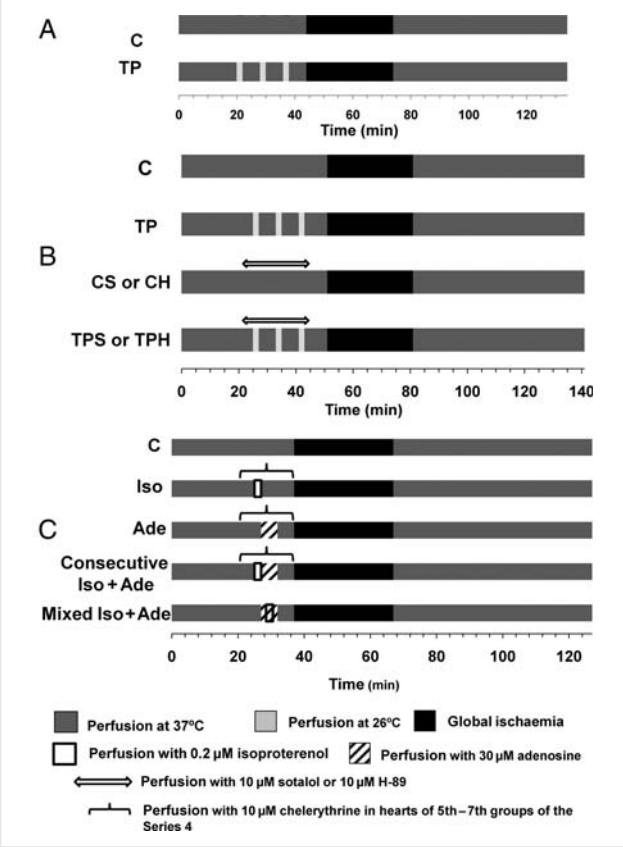

Figure 1 Outline of the protocols used in the experiments. (*A*) Series 1. TP, temperature preconditioning; C, control. (*B*) Series 2. CS and CH, control hearts perfused with 10 μM sotalol or H-89, respectively. TPS and TPH, TP hearts perfused with 10 μM sotalol or H-89, respectively. (*C*) Series 3 and 4. Iso, hearts perfused with 0.2 μM isoproterenol (2 min, 37°C) + 10 min washout; Ade, hearts perfused with 30 μM adenosine (5 min, 37°C) + 5 min washout; consecutive Iso + Ade, hearts perfused with isoproterenol then adenosine + 5 min washout; mixed Iso + Ade, 2 min perfusion with 0.2 μM isoproterenol during 5 min perfusion with 30 μM adenosine followed by 5 min washout. In Series 4, Iso, Ade, and the consecutive Iso + Ade treatments were also combined with 10 μM chelerythrine infusion started 5 min before perfusion with isoproterenol or adenosine and completed at the end of pre-ischaemia.

2.1.3 Assays

PKA and PKC activities and cAMP concentration were determined in freeze-clamped heart powders using kits supplied by Sigma (cAMP) and Promega (PKA and PKC) according to the manufacturer's instructions. The assays of PKA and PKC activity rely on a change in charge of the fluorescent PepTag® A1 and PepTag® C1 peptides from +1 to −1 following phosphorylation. Bands were visualized under UV light and the ratio of fluorescence intensity of phosphorylated to non-phosphorylated peptide was quantified using AlphaInotech ChemiImager 4400 with AlphaEase v5.5 software. The phosphorylation of Akt and GSK3α/β was determined in freeze-clamped, powdered hearts by a method based on that of Hausenloy *et al.*[8] using western blotting with antibodies (Cell Signaling Technology) against phosphorylated and total Akt and GSK3α/β. The ratio of the band intensity for phosphoprotein to total protein was used as a measure of phosphorylation state.

The assay of glycogen in freeze-clamped pre-ischaemic hearts was performed using α-glycosidase hydrolysis with measurement of the released glucose performed spetrophotometrically (340 nm) as described previously.[9]

Mitochondria were isolated as described in Supplementary Methods and measurement of MPTP opening performed in de-energized conditions at 30°C as described previously[3] using the decrease in light scattering (A_{520}) that accompanies mitochondrial swelling following addition of 100 μM Ca^{2+}.

Protein carbonyls were determined in frozen mitochondria following derivatization with dinitrophenylhydrazine (Chemicon International, Chandlers Ford, UK) and western blotting with anti-dinitrophenyl antibodies (Sigma) exactly as described previously.[10]

Further details may be found in Supplementary Methods.

2.2 Statistical analysis

Data are presented as mean ± SEM. Statistical significances of the differences between groups were evaluated using Student's t-test (Series 1) or one-way ANOVA followed by Tukey's multiple comparison post hoc test (Series 2–4) using GraphPad Prism v5.0 software. Differences were considered significant where $P < 0.05$.

3. Results

3.1 PKA activity and Akt/GSK3 phosphorylation following TP (Series 1)

In *Table 1*, we show that during reperfusion, recovery of LVDP and RPP in TP hearts was two-fold greater than for control hearts with a 60% increase in the time derivatives of LV pressure. Protection against necrotic damage (LDH release) during the first 15 min of reperfusion showed a similar pattern to the recovery of haemodynamic function. *Figure 2* shows that after the TP protocol, the tissue concentration of cAMP was significantly increased (*Figure 2B*) as was PKA activity (*Figure 2A*). However, neither GSK3α/β (*Figure 2C* and *E*) nor Akt (*Figure 2D* and *F*) showed any change in phosphorylation following the TP protocol or after 15 min reperfusion.

3.2 β-Adrenergic stimulation of PKA is required for PKC activation and cardioprotection by TP (Series 2)

The role of β-adrenergic stimulation and PKA activation in TP was investigated using the β-adrenergic blocker (±)-sotalol[11] and the PKA inhibitor H-89.[12] In preliminary experiments (data not shown), we found that both 10 μM sotalol and 10 μM H-89 completely and reversibly abolished the increase in haemodynamic function induced by isoproterenol (0.2 μM).

Prior to ischaemia, the RPP of sotalol-treated hearts (TPS group) was significantly lower than untreated hearts during the first and third hypothermic episodes, and sotalol also suppressed the rise of HR during the subsequent normothermia leading to a smaller increase in RPP (*Figure 3A*). H-89 (TPH group) also decreased LVDP, although HR of these hearts was higher than in the TPS hearts in all three normothermic episodes (Supplementary material online, *Table S1*). The combined effect was a lower RPP in TPH hearts relative to TP (*Figure 3A*), but less so than in TPS hearts. H-89 also blocked the increase in PKC activity seen in TP hearts without affecting PKC activity in control hearts (*Figure 3B*).

Neither sotalol (CS) nor H-89 (CH) affected recovery of LVDP or RPP in control hearts but they did attenuate (sotalol, TPS group) or prevent (H-89, TPH group) the increased haemodynamic recovery seen in TP hearts. The effects of sotalol and H-89 on haemodynamic function were matched by their ability to reduce or abolish the protection TP offers against necrosis (LDH release) (*Table 2*).

3.3 The consecutive pharmacological activation of PKA and PKC induced powerful cardioprotection (Series 3 and 4)

3.3.1 Pre-ischaemic effects

Adenosine reduced RPP by ∼20% ($P < 0.05$) with subsequent gradual return of this parameter to the initial value, whereas perfusion with

Table 1 Effects of TP on haemodynamic function and LDH release from the hearts during reperfusion

Parameters	Pre-ischaemia ($n = 27$)	Reperfusion		
			Control ($n = 15$)	TP ($n = 12$)
LVDP (mmHg)	80.7 ± 3.9	% of initial values	30.4 ± 3.6	69.8 ± 7.7***
HR (b.p.m.)	302.0 ± 5.2		100.4 ± 3.3	97.6 ± 2.4
RPP (mmHg b.p.m)	24 273 ± 1164		31.3 ± 4.2	67.0 ± 6.7***
+d*P*/d*t* (mmHg/s)	3697 ± 145		45.5 ± 3.5	71.6 ± 6.2**
−d*P*/d*t* (mmHg/s)	2984 ± 132		52.2 ± 4.3	84.2 ± 5.8***
LDH (mU/mL perfusate)	1.9 ± 0.2	5 min	24.7 ± 3.3	13.6 ± 2.1*
		10 min	25.0 ± 4.8	13.2 ± 2.0*
		15 min	24.5 ± 4.2	11.8 ± 2.2*

Haemodynamic function was determined prior to ischaemia and after 60 min reperfusion. LDH release was measured prior to ischaemia and during the first 15 min of reperfusion. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

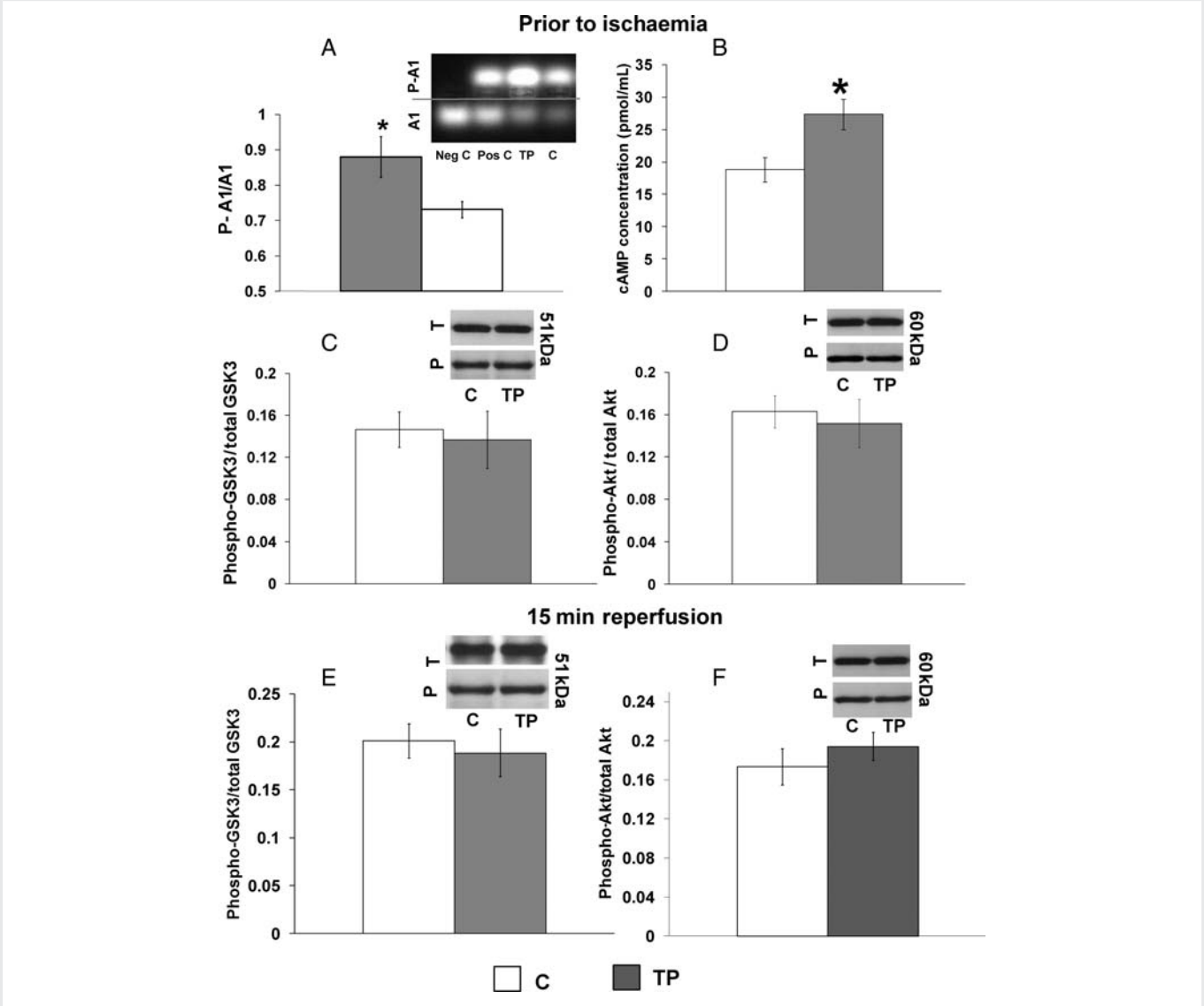

Figure 2 Effect of TP on PKA activity, cAMP concentration, and Akt/GSK3 phosphorylation. (*A*) PKA activity was measured using the PepTag® assay (Promega) and expressed as a ratio of fluorescence intensity of phosphorylated and non-phosphorylated PepTag® A1 peptide (P-A1 and A1, respectively). (Inset) A representative gel containing A1 and P-A1; Neg C, negative control; Pos C, positive control (PepTag® A1 peptide phosphorylated by the PKA catalytic subunit). (*B*) cAMP concentration was determined using a direct enzyme immunoassay kit (Sigma). (*C*–*F*) GSK3 (*C* + *E*) and Akt (*D* + *F*) phosphorylation were determined prior to ischaemia (*C* + *D*) or after 15 min reperfusion (*E* + *F*) by western blotting using the ratio of band intensity of phosphorylated to total protein. Eight hearts each of control (C) and temperature preconditioning (TP) groups were used for all parameters. *$P < 0.05$ vs. control. Inset in each of (*C*–*F*): representative blots of phosphorylated (P) and total (T) GSK3 and Akt.

isoproterenol increased RPP 2.5-fold. When hearts perfused with isoproterenol were switched to adenosine, RPP was reduced to 60% of the initial value ($P < 0.01$) and was significantly lower than in adenosine-treated hearts ($P < 0.01$). At the end of pre-ischaemia, this parameter was still slightly decreased in hearts of the consecutive isoproterenol + adenosine group ($P < 0.05$ vs. initial value) (*Figure 4A*). Perfusion with isoproterenol but not adenosine also reduced the glycogen content of the hearts by >50%. There was no additive effect of adenosine on the response to isoproterenol (*Figure 4B*). PKC activity, measured immediately after perfusion with isoproterenol or adenosine, was significantly higher in all three groups of isoproterenol and adenosine-treated hearts (*Figure 4C*).

3.3.2 Cardioprotection is associated with inhibition of protein carbonylation and MPTP opening on reperfusion

Treatment of hearts with either isoproterenol or adenosine alone improved haemodynamic function recovery after 30 min global ischaemia and 60 min reperfusion (*Table 3*); the recovered LVDP and RPP reached twice the values of control reperfused hearts. However, consecutive treatment of hearts with isoproterenol followed by adenosine resulted in a complete recovery of haemodynamic function accompanied by the lowest LDH release (*Table 3*).

Haemodynamic function recovery of hearts treated simultaneously (as opposed to consecutively) with the mixture of adenosine and isoproterenol (*Table 4*) was similar to hearts treated with isoproterenol

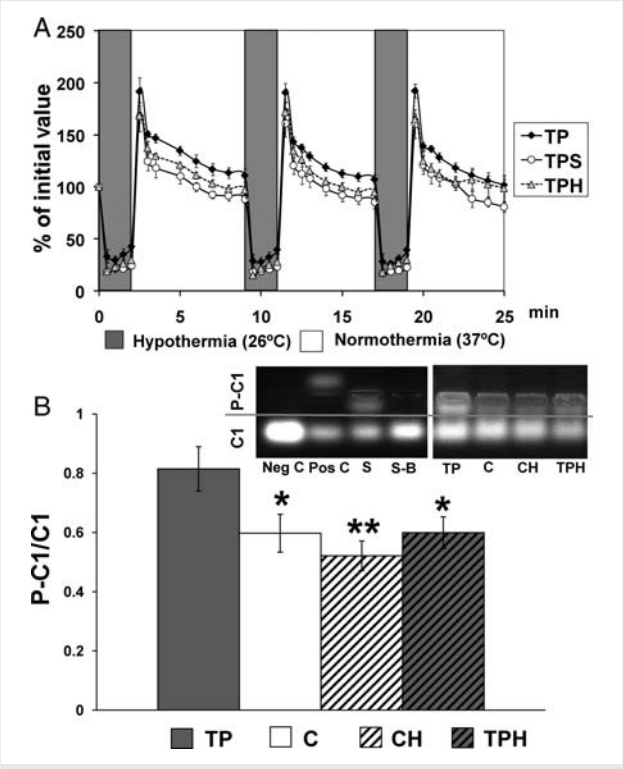

Figure 3 Effect of sotalol and H-89 on RPP and PKC activity of control and TP hearts prior to ischaemia. (*A*) Mean data for RPP measured during hypothermic and normothermic perfusion in TP ($n = 8$), TP + 10 μM sotalol (TPS; $n = 6$), and TP + 10 μM H-89 (TPH; $n = 6$) hearts. RPP values for TPH and TPS groups were significantly lower ($P < 0.05$) than TP during all three episodes of normothermic perfusion. (*B*) Mean data for PKC activity measured in eight hearts each of control (C), TP, control + 10 μM H-89 (CH), and TPH groups. PKC activity was measured using non-radioactive PepTag® assay and is expressed as a ratio of fluorescence intensity of phosphorylated and non-phosphorylated peptide. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TP. (Inset) Representative gels containing non-phosphorylated and phosphorylated PepTag® C1 peptide (C1 and P-C1, respectively). Specificity of the PepTag® C1 peptide to PKC was confirmed by its reaction with PKC control enzyme (Pos C, positive control) and a heart sample (S). No phosphorylated peptide was found without the control enzyme (Neg C, negative control) or with the boiled heart sample (S-B).

or adenosine alone and significantly lower than for consecutive treatment. The PKC inhibitor chelerythrine completely abolished the improvement of haemodynamic function recovery in adenosine-treated hearts and considerably reduced the beneficial effect of the consecutive isoproterenol and adenosine treatment but had no significant effect on the recovery of isoproterenol-treated hearts. LDH release was the lowest in hearts with the consecutive treatment (*Tables 3* and *4*); in hearts with the simultaneous isoproterenol and adenosine treatment this parameter was not significantly less than controls (*Table 4*). In hearts treated with the combination of adenosine and chelerythrine, LDH release was the same as control.

Both isoproterenol and adenosine reduced Ca^{2+}-induced mitochondria swelling (MPTP opening) after 30 min global ischaemia to 17 and 41% of control ischaemic values, respectively, whereas the sequential treatment with the two agents reduced swelling to 5%

Table 2 Effects of sotalol and H-89 on the protective effects of TP on haemodynamic function and LDH release during reperfusion

Parameters	Pre-ischaemia ($n = 40$)	Reperfusion	Control ($n = 8$)	TP ($n = 8$)	CS ($n = 6$)	TPS ($n = 6$)	CH ($n = 6$)	TPH ($n = 6$)
LVDP (mmHg)	74.0 ± 1.9	% of initial values	37.5 ± 4.5	76.3 ± 4.7***	45.2 ± 12.4$^{\#}$	59.0 ± 8.6$^{*,\#}$	43.5 ± 10.6$^{\#}$	37.4 ± 6.6$^{\#\#}$
HR (b.p.m.)	296.5 ± 4.5		100.8 ± 1.4	99.7 ± 2.2	102.3 ± 2.9	96.6 ± 2.9	98.8 ± 3.0	103.9 ± 2.8
RPP (mmHg b.p.m.)	21 833 ± 520		37.9 ± 4.5	75.7 ± 4.0***	46.3 ± 12.6$^{\#}$	57.1 ± 8.7$^{\#}$	42.9 ± 9.8$^{\#}$	39.0 ± 7.3$^{\#\#}$
+dP/dt (mmHg/s)	3598 ± 82		32.2 ± 3.3	76.8 ± 5.2***	40.8 ± 8.3$^{\#\#}$	48.1 ± 5.9$^{*,\#}$	37.8 ± 7.4$^{\#\#}$	51.9 ± 8.6$^{\#}$
−dP/dt (mmHg/s)	2905 ± 80		37.5 ± 3.6	78.8 ± 8.0***	50.4 ± 10.7$^{\#}$	47.5 ± 4.9$^{\#\#}$	49.7 ± 9.9$^{\#}$	54.1 ± 5.4$^{*,\#}$
LDH (mU/mL perfusate)	3.1 ± 0.3	5 min	19.8 ± 3.3	11.2 ± 0.7*	15.3 ± 1.0$^{\#}$	14.1 ± 1.4	22.9 ± 3.9$^{\#}$	17.8 ± 3.0
		10 min	26.4 ± 4.6	12.1 ± 1.6*	23.7 ± 4.8$^{\#}$	16.7 ± 2.4	28.5 ± 5.6$^{\#}$	22.5 ± 4.8$^{\#}$
		15 min	22.9 ± 2.3	10.3 ± 2.0**	21.7 ± 4.9$^{\#}$	17.8 ± 3.0	22.5 ± 4.8$^{\#}$	23.7 ± 5.3$^{\#}$

Haemodynamic function was assessed prior to ischaemia and after 60 min reperfusion. LDH release was measured prior to ischaemia and during the first 15 min of reperfusion. CS and TPS represent sotalol (10 μM) alone or in combination with TP. CH and TPH represent H-89 (10 μM) alone or in combination with TP.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. TP.

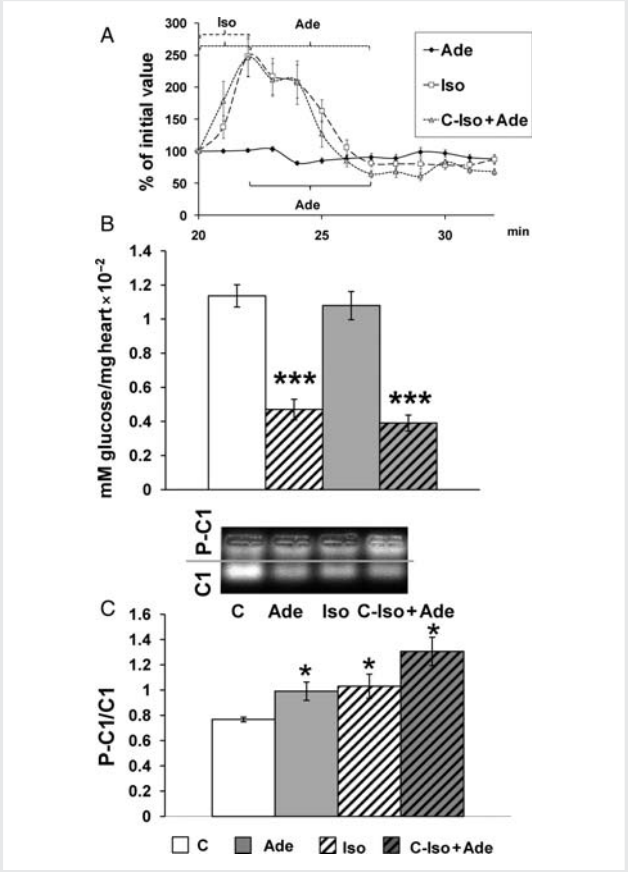

Figure 4 Effect of isoproterenol and adenosine on RPP, glycogen content, and PKC activity in the hearts prior to ischaemia. (*A*) Mean data for RPP in 0.2 μM isoproterenol (Iso; $n = 7$), 30 μM adenosine (Ade; $n = 8$), and consecutive isoproterenol + adenosine (C-Iso + Ade; $n = 11$) hearts measured during pre-ischaemia following the equilibration period. Isoproterenol significantly increased and adenosine reduced RPP compared with control hearts (C). The decrease in RPP was significantly greater in the C-Iso + Ade hearts than in the Ade hearts ($P < 0.05$) starting from 27 min pre-ischaemia. (*B*) Mean data for glycogen content in six hearts each of control C, Iso, Ade, and C-Iso + Ade groups. ***$P < 0.001$ vs. control. (*C*) Mean data for PKC activity in six each of C, Iso, Ade, and C-Iso + Ade groups measured in hearts prior to ischaemia. *$P < 0.05$ vs. control. (Inset) A representative gel containing non-phosphorylated and phosphorylated PepTag® C1 peptide (C1 and P-C1, respectively).

indicating almost total prevention of MPTP opening (*Figure 5A*). Parallel measurements of mitochondrial protein carbonylation showed that only the consecutive isoproterenol + adenosine group gave a significant reduction in this parameter (*Figure 5B*).

4. Discussion

4.1 The signal transduction pathways of TP

4.1.1 Role of PKA activation

Here, we report that a β-adrenergic-mediated rise in cAMP and subsequent activation of PKA is important for TP-induced cardioprotection which is consistent with our earlier observation[2] that the switch from hypothermic perfusion to normothermia during TP caused a fast and significant augmentation of haemodynamic function (see also

Table 3 Effects of isoproterenol and adenosine on haemodynamic function and LDH release during reperfusion

Parameters	Pre-ischaemia ($n = 28$)	Reperfusion	Control ($n = 8$)	Ade ($n = 8$)	Iso ($n = 6$)	C-Iso + Ade ($n = 6$)
LVDP (mmHg)	74.4 ± 3.7	% of initial values	24.1 ± 2.9	50.5 ± 7.5*,##	56.3 ± 7.6**,##	102.7 ± 11.0**,*
HR (b.p.m.)	296.8 ± 5.6		98.7 ± 2.7	98.8 ± 2.3	98.1 ± 2.1	100.3 ± 1.8
RPP (mmHg b.p.m.)	22 700 ± 1128		24.2 ± 3.3	49.3 ± 6.8*,##	56.0 ± 8.5*,#	102.8 ± 11.0**,*
+dP/dt (mmHg/s)	2752 ± 84		27.2 ± 3.5	51.5 ± 7.1##	55.6 ± 7.4*,##	93.5 ± 11.6**,*
−dP/dt (mmHg/s)	2049 ± 71		29.8 ± 3.2	54.1 ± 5.9#	56.3 ± 6.7*,#	96.2 ± 14.3**,*
LDH (mU/mL perfusate)	2.0 ± 0.3	5 min	13.7 ± 2.0	10.2 ± 2.0	10.2 ± 0.9	6.5 ± 1.8*
		10 min	16.0 ± 2.8	12.0 ± 2.4	10.7 ± 1.1	6.5 ± 2.2*
		15 min	15.5 ± 2.4	12.2 ± 2.3#	8.8 ± 1.4*	5.3 ± 1.5**

Haemodynamic function was assessed prior to ischaemia and after 60 min reperfusion. LDH release was measured prior to ischaemia and during the first 15 min of reperfusion. Ade, adenosine; Iso, isoproterenol; C-Iso + Ade, consecutive treatment with Iso and Ade.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.
#$P < 0.05$, ##$P < 0.01$ vs. C-Iso + Ade.

Table 4 Comparison of the effects of chelerythrine and consecutive and simultaneous perfusion with isoproterenol and adenosine on haemodynamic function and LDH release from the hearts during reperfusion

Parameters	Pre-ischaemia ($n = 46$)	Reperfusion							
			Control ($n = 8$)	M-Iso + Ade ($n = 6$)	C-Iso + Ade ($n = 5$)	Che + (C-Iso + Ade) ($n = 7$)	Iso ($n = 5$)	Che + Iso ($n = 5$)	Ade ($n = 5$)	Che + Ade ($n = 5$)
LVDP (mmHg)	79.3 ± 3.0	% of initial values	23.4 ± 4.1	59.4 ± 7.2$^{**,\#\#}$	101.8 ± 5.8***	60.1 ± 3.8***,†††	58.7 ± 8.5$^{*,\#\#}$	47.0 ± 5.7$^{*,\#\#\#}$	47.5 ± 1.7$^{*,\#\#\#}$	25.7 ± 1.6$^{\dagger,\#\#\#}$
HR (b.p.m.)	283.3 ± 5.1		99.6 ± 6.5	97.9 ± 3.1	98.3 ± 1.9	93.5 ± 3.3	92.2 ± 5.3	96.6 ± 2.1	98.3 ± 3.1	98.0 ± 2.2
RPP (mmHg b.p.m.)	22 411 ± 903		22.7 ± 3.5	57.8 ± 6.8$^{***,\#\#\#}$	100.2 ± 6.7***	55.7 ± 2.7**,†††	55.0 ± 9.9$^{*,\#\#}$	45.2 ± 5.1$^{*,\#\#\#}$	46.3 ± 1.3 * $^{\#\#\#}$	25.4 ± 2.2$^{\dagger,\#\#\#}$
+d*P*/d*t* (mmHg/s)	2703 ± 84		23.1 ± 2.7	59.8 ± 6.8$^{**,\#\#\#}$	117.5 ± 5.5***	60.8 ± 5.1***,†††	60.4 ± 9.1$^{*,\#\#}$	49.4 ± 6.5$^{*,\#\#\#}$	46.8 ± 3.7$^{*,\#\#\#}$	26.1 ± 0.6$^{\dagger,\#\#\#}$
−d*P*/d*t* (mmHg/s)	2150 ± 103		24.3 ± 3.4	52.9 ± 5.8$^{**,\#\#\#}$	99.0 ± 5.2***	43.1 ± 5.1*,†††	52.2 ± 8.3*	39.5 ± 8.4$^{\#\#\#}$	43.5 ± 1.0$^{*,\#\#\#}$	33.5 ± 2.7$^{\dagger,\#\#\#}$
LDH (mU/mL perfusate)	1.3 ± 0.1	5 min	10.3 ± 1.8	7.8 ± 2.0	3.6 ± 0.8**	7.4 ± 1.9	8.8 ± 2.6	8.5 ± 0.7	9.2 ± 2.5	13.4 ± 2.3$^{\#\#}$
		10 min	14.5 ± 3.1	10.7 ± 3.0	6.1 ± 1.4*	7.2 ± 3.0	7.7 ± 2.6	8.3 ± 1.2	10.8 ± 2.5	13.3 ± 2.3$^{\#}$
		15 min	12.8 ± 3.1	10.1 ± 2.2$^{\#}$	3.8 ± 1.3*	6.0 ± 2.0	5.2 ± 1.2	7.2 ± 1.3	7.7 ± 2.5	12.1 ± 2.0$^{\#\#}$

Haemodynamic function was determined prior to ischaemia and after 60 min reperfusion. LDH release was measured prior to ischaemia and during the first 15 min of reperfusion. Iso, isoproterenol; Ade, adenosine; C-Iso + Ade, consecutive treatment with Iso and Ade; M-Iso + Ade, mixed Iso and Ade treatment; Che, chelerythrine.

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. C-Iso + Ade.

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ of a group of hearts treated with Che vs. a corresponding group non-treated with Che. Che (10 μM) had no effect on haemodynamic function and LDH release during reperfusion in control hearts.[2]

Figure 5 Effect of isoproterenol and adenosine on Ca^{2+}-induced MPTP opening (*A*) and protein carbonylation (*B*). (*A*) Mean data for the rate of Ca^{2+}-induced mitochondria swelling in C ($n = 8$), Iso ($n = 7$), Ade ($n = 7$), and C-Iso + Ade ($n = 9$) groups of hearts. Mitochondria were isolated after 30 min global ischaemia and MPTP opening determined under de-energized conditions as described under Section 2. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. C, $^{\#\#}P < 0.01$ vs. Iso + Ade. (*B*) Protein carbonylation measured by western blotting in mitochondria isolated from eight hearts each of non-ischaemic control (CP) and C, Iso, Ade, and C-Iso + Ade groups after 30 min global ischaemia. A representative blot is shown plus mean data for each condition. ND, non-derivatized control used to correct for non-specific binding. Further details are given in Supplementary Methods. $^{**}P < 0.01$ vs. control.

Figure 3A; see Supplementary material online, *Table S1*). These changes of contractility might reflect the increased accumulation of intracellular calcium that accompanies hypothermic perfusion[13] and our current data suggest that endogenous β-adrenergic agonists may cause this through their known effects on intracellular calcium homeostasis. Thus, we have shown that myocardial cAMP concentration and PKA activity were increased at the end of TP protocol (*Figure 2A* and *B*) and that the non-selective β-adrenergic blocker sotalol and the PKA inhibitor H-89 attenuate the cardioprotective effects of TP. Both agents reduced the augmentation of haemodynamic function during the TP protocol (see Supplementary material

online, *Table S1*; *Figure 3A*), whereas H-89 completely abolished and sotalol partially abolished the TP-mediated improvement of haemodynamic function and reduction of LDH release during reperfusion (*Table 2*). These data suggest that β-adrenergic stimulation is only partially responsible for PKA activation during TP protocol and that another pathway, perhaps ROS-mediated,[14] may also be involved. Indeed, we have shown previously that the free radical scavenger *N*-(2-mercaptopropionyl)-glycine (300 μM) applied during pre-ischaemia abolishes cardioprotection by TP as it does for IP.[2] Furthermore, Stowe and colleagues[15] have reported that hypothermia moderately enhances superoxide concentration in myocardium.

Interestingly, it has been shown by others that β-adrenergic stimulation can be considered as a trigger of IP[4] and that repeated stimulation with norepinephrine or isoproterenol mimics IP.[16] Proposed mechanisms for cardioprotection by β-adrenergic activation of PKA include β-adrenergic desensitization[4] and attenuation of calpain-mediated degradation pathways.[12]

A major problem with using protein kinase inhibitors to dissect signal transduction pathways is their lack of specificity.[17] Although H-89 is a potent PKA inhibitor, it can inhibit other kinases including Akt.[17] The Akt–GSK3 pathway has been implicated in cardioprotection by IP,[18] although our own data[3] and that of other[19] have questioned the central role of this pathway prior to ischaemia and we were unable to detect any change in phosphorylation of either protein following the TP protocol (*Figure 2C* and *D*). Hausenloy *et al.*[8] showed IP-induced Akt phosphorylation at 15 min of reperfusion following prolonged ischaemia, but we were also unable to detect any changes in Akt or GSK3 phosphorylation by TP at 15 min reperfusion (*Figure 2E* and *F*). However, we cannot completely exclude involvement of this pathway in TP because phosphorylation could be transient; although in studies where Akt activation and GSK3 inhibition were found to be important for cardioprotection, phosphorylation of these kinases was very consistent and significant during preischaemia[20] and reperfusion.[8,21] Thus, our data do not support a major role of Akt and GSK3 phosphorylation in the TP signalling mechanism.

4.1.2 Sequential PKA and PKC activation during TP and pharmacologically induced consecutive PKA/PKC activation

We have previously shown that PKC activation is critical for TP-mediated cardioprotection,[2] and here, we demonstrate that the PKA inhibitor H-89, which itself has little effect on PKC activity,[17] is able to prevent both this PKC activation (*Figure 3B*) and cardioprotection (*Table 2*), implying that PKA activation is upstream of PKC activation in the TP signalling pathway. Our data further support this finding. Thus, treatment of rat hearts consecutively with the β-adrenergic agonist isoproterenol and then adenosine, to activate PKC, resulted in extremely potent cardioprotection that considerably exceeded the protection afforded by either agent alone (*Table 3*) or added simultaneously (*Table 4*) and allowed hearts to recover completely after 30 min normothermic (37°C) global ischaemia. Thus, the consecutive activation of these enzymes (PKA followed by PKC activation) is of critical importance in terms of cardioprotection.

4.2 Mechanisms underlying the cardioprotective effect of sequential PKA/PKC activation

4.2.1 Glycogen breakdown

The decreased glycogen content found in isoproterenol-treated hearts (*Figure 4B*) could contribute to the observed cardioprotective effect. Thus, it has been reported that glycogen depletion of rat hearts by anoxic perfusion prior to global ischaemia significantly improved recovery of ventricular function during reperfusion, whereas lactate accumulation was harmful for the heart.[22] Others showed that IP is associated with glycogen depletion leading to less anaerobic glycolysis during the subsequent prolonged ischaemia, and hence reduced accumulation of lactate and H^+. This causes a smaller decrease in intracellular pH during ischaemia and thus less compensatory increases in intracellular Na^+ and Ca^{2+}.[23] The decreased calcium loading will reduce the likelihood that the MPTP opens which might partially explain the observed cardioprotection.[24] However, reduced glycogen content alone cannot entirely account for the cardioprotection in our experiments since this parameter was similar in hearts treated with isoproterenol alone or with adenosine; yet, the combined treatment gave much better protection. Our data suggest that the additional factor is activation of PKC.

4.2.2 Involvement of PKC in cardioprotection

It has been shown that isoproterenol can augment the negative inotropic effect of adenosine[25] which we also observed in the pre-ischaemic phase of the combined treatment (*Figure 4A*). This would be consistent with PKA activation of PKC, and in support of this, our data show that perfusion with the β-adrenergic agonist isoproterenol does increase PKC activity in the heart (*Figure 4C*). Stimulation of β-adrenergic receptors and PKA activates ROS production by mitochondria[26] and promotes accumulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$).[27] Meanwhile, it has been found that ROS may trigger PKC activation[28] and increase in $[Ca^{2+}]_i$ could also activate PKC via direct Ca^{2+}-dependent activation or via a G-protein activated by Ca^{2+}-dependent phospholipase C.[29]

The cardioprotective effect of PKC activation (primarily PKCε) is well established[7] and we have also shown recently that urocortin-induced reduction of oxidative stress is mediated by PKC resulting in MPTP inhibition during reperfusion.[30] How this is achieved remains unclear. It has been reported that PKCε may phosphorylate the voltage-dependent anion channel in the outer mitochondrial membrane or prevent binding of cyclophilin D to adenine nucleotide translocase which results in inhibition of MPTP,[31] although our own data failed to detect these changes in IP.[3] PKC may also phosphorylate the BH3-only protein Bad[32] which increases the availability of Bcl-2 for antioxidant and anti-apoptotic functions.[33] Whatever the mechanism, it appears that PKCε activation prevents ROS production and MPTP opening during ischaemia and reperfusion.

We have previously found that the PKC inhibitor chelerythrine abrogated TP-induced cardioprotection,[2] and here, we show (*Table 4*) that chelerythrine completely abolished the protective effect of adenosine and considerably reduced cardioprotection afforded by the consecutive isoproterenol/adenosine treatment. However, chelerythrine had little effect on the protective effect of isoproterenol. These results suggest that PKA-induced cardioprotection in our experiments did not rely exclusively on PKC activation but was associated with other mechanisms including glycogen depletion prior to ischaemia. Our data also show that the strong protective effect of the consecutive isoproterenol/adenosine treatment was rather a result of the synergic action of both PKA and PKC than PKC being the only effector in the signalling mechanisms of this pharmacological treatment.

4.2.3 Decreased oxidative stress and reduced MPTP opening

We demonstrated previously that protection by TP involves inhibition of MPTP opening.[2] Here, we show that consecutive treatment of the heart with isoproterenol and adenosine also dramatically reduced calcium-induced mitochondria swelling, an indicator of MPTP opening (*Figure 5A*). Treatment with isoproterenol or adenosine alone also gave a significant, but smaller, reduction in calcium-induced mitochondria swelling. This is of interest because it was demonstrated more than 30 years ago that mitochondria isolated from livers treated with glucagon, dibutyryl-cAMP or α-adrenergic agonists (PKC activating) retained accumulated calcium for longer than those from control livers.[34–36] This increase in 'calcium retention time' is now known to reflect an inhibition of MPTP opening and thus it appears likely that a similar cAMP-dependent protective mechanism to that seen in the heart also operates in liver.

For both IP and TP, inhibition of the MPTP in mitochondria isolated at the end of ischaemia or during reperfusion correlates with a decreased oxidative stress as reflected in protein carbonylation,[2,3] and here, we show that the powerful protective effect of the sequential isoproterenol + adenosine treatment was also accompanied by a significant decrease in protein carbonylation (*Fig 5B*). Treatment with each agent on its own also showed a slight reduction in protein carbonylation but this was not statistically significant. No published data are available on the effects of glucagon or α-adrenergic agonists on liver mitochondrial protein carbonylation, but glucagon was found to decrease mitochondrial lysophospholipid accumulation[37] consistent with reduced lipid peroxidation,[38] another indicator of oxidative stress. Thus, it is possible that the inhibition of MPTP opening by cAMP-dependent mechanisms in liver, as well as in TP and particularly isoproterenol + adenosine-treated hearts, involves a decrease in oxidative stress.

4.3 Conclusions

The novel findings of our study are as follows. First, PKA activation, like PKC activation, is a very important link in the signalling mechanism of TP with PKA activation being upstream of PKC activation and mediated in part by β-adrenergic stimulation. Secondly, pharmacologically induced consecutive PKA and PKC activation provides potent cardioprotection. Thirdly, this cardioprotection is associated with depletion of myocardial glycogen prior to ischaemia which may lead to less calcium loading during ischaemia. This, together with the reduction of oxidative stress during ischaemia, will lead to less MPTP opening and thus better recovery during reperfusion. This pharmacological strategy may represent a promising protocol for heart protection during prolonged ischaemia and reperfusion such as in open heart surgery or transplantation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

The authors would like to thank Professor Saadeh Suleiman, Elinor Griffiths, and Philippe Pasdois for helpful discussions and Marieangela C. Wilson for technical advice.

Conflict of interest: none declared.

Funding

This work was supported by a grant from the British Heart Foundation (PG/07/080/23613). Funding to pay the Open Access publication charges for this article was provided by British Heart Foundation.

References

1. Depre C, Taegtmeyer H. Metabolic aspects of programmed cell survival and cell death in the heart. *Cardiovasc Res* 2000;**45**:538–548.
2. Khaliulin I, Clarke SJ, Lin H, Parker JE, Suleiman MS, Halestrap AP. Temperature preconditioning of isolated rat hearts—a potent cardioprotective mechanism involving a reduction in oxidative stress and inhibition of the mitochondrial permeability transition pore. *J Physiol* 2007;**581**:1147–1161.
3. Clarke SJ, Khaliulin I, Das M, Parker JE, Heesom KJ, Halestrap AP. Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation. *Circ Res* 2008;**102**:1082–1090.
4. Lochner A, Genade S, Tromp E, Podzuweit T, Moolman JA. Ischemic preconditioning and the β-adrenergic signal transduction pathway. *Circulation* 1999;**100**:958–966.
5. Yellon DM, Dana A. The preconditioning phenomenon: a tool for the scientist or a clinical reality? *Circ Res* 2000;**87**:543–550.
6. Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. *J Physiol* 2003;**549**:513–524.
7. Cohen MV, Downey JM. Adenosine: Trigger and mediator of cardioprotection. *Basic Res Cardiol* 2008;**103**:203–215.
8. Hausenloy DJ, Mocanu MM, Yellon DM. Cross-talk between the survival kinases during early reperfusion: its contribution to ischemic preconditioning. *Cardiovasc Res* 2004;**63**:305–312.
9. Passonneau JV, Lauderdale VR. A comparison of three methods of glycogen measurement in tissues. *Anal Biochem* 1974;**60**:405–412.
10. Khaliulin I, Schneider A, Houminer E, Borman JB, Schwalb H. Apomorphine prevents myocardial ischemia/reperfusion-induced oxidative stress in the rat heart. *Free Radic Biol Med* 2004;**37**:969–976.
11. Guérard NC, Traebert M, Suter W, Dumotier BM. Selective block of iks plays a significant role in map triangulation induced by ikr block in isolated rabbit heart. *J Pharmacol Toxicol Methods* 2008;**58**:32–40.
12. Inserte J, Garcia-Dorado D, Ruiz-Meana M, Agullo L, Pina P, Soler-Soler J. Ischemic preconditioning attenuates calpain-mediated degradation of structural proteins through a protein kinase A-dependent mechanism. *Cardiovasc Res* 2004;**64**:105–114.
13. Stowe DF, Fujita S, An J, Paulsen RA, Varadarajan SG, Smart SC. Modulation of myocardial function and [Ca^{2+}] sensitivity by moderate hypothermia in guinea pig isolated hearts. *Am J Physiol Heart Circ Physiol* 1999;**277**:H2321–H2332.
14. Brennan JP, Bardswell SC, Burgoyne JR, Fuller W, Schroder E, Wait R *et al*. Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. *J Biol Chem* 2006;**281**:21827–21836.
15. Camara AK, Riess ML, Kevin LG, Novalija E, Stowe DF. Hypothermia augments reactive oxygen species detected in the guinea pig isolated perfused heart. *Am J Physiol Heart Circ Physiol* 2004;**286**:H1289–H1299.
16. Asimakis GK, Inners-McBride K, Conti VR, Yang CJ. Transient beta adrenergic stimulation can precondition the rat heart against postischaemic contractile dysfunction. *Cardiovasc Res* 1994;**28**:1726–1734.
17. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H *et al*. The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007;**408**:297–315.
18. Murphy E. Primary and secondary signaling pathways in early preconditioning that converge on the mitochondria to produce cardioprotection. *Circ Res* 2004;**94**:7–16.
19. Heusch G. No risk, no . . . cardioprotection? A critical perspective. *Cardiovasc Res* 2009;**84**:173–175.
20. Krieg T, Qin Q, McIntosh EC, Cohen MV, Downey JM. Ach and adenosine activate pi3-kinase in rabbit hearts through transactivation of receptor tyrosine kinases. *Am J Physiol Heart Circ Physiol* 2002;**283**:H2322–H2330.
21. Fang N-X, Yao Y-T, Shi C-X, Li L-H. Attenuation of ischemia–reperfusion injury by sevoflurane postconditioning involves protein kinase B and glycogen synthase kinase 3 beta activation in isolated rat hearts. *Mol Biol Rep*; doi: 10.1007/s11033-010-0030-5. Published online ahead of print 10 March 2010.
22. Neely JR, Grotyohann LW. Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circ Res* 1984;**55**:816–824.
23. Cross HR, Opie LH, Radda GK, Clarke K. Is a high glycogen content beneficial or detrimental to the ischemic rat heart? A controversy resolved. *Circ Res* 1996;**78**:482–491.
24. Halestrap AP, Pasdois P. The role of the mitochondrial permeability transition pore in heart disease. *Biochim Biophys Acta* 2009;**1787**:1402–1415.
25. El-Ani D, Zimlichman R, Mashiach Y, Shainberg A. Adenosine and TNF-α exert similar inotropic effect on heart cultures, suggesting a cardioprotective mechanism against hypoxia. *Life Sci* 2007;**81**:803–813.
26. Nagasaka S, Katoh H, Niu CF, Matsui S, Urushida T, Satoh H *et al*. Protein kinase A catalytic subunit alters cardiac mitochondrial redox state and membrane potential via the formation of reactive oxygen species. *Circ J* 2007;**71**:429–436.

27. Wallukat G. The β-adrenergic receptors. *Herz* 2002;**27**:683–690.
28. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF. Reactive oxygen species precede the epsilon isoform of protein kinase c in the anesthetic preconditioning signaling cascade. *Anesthesiology* 2003;**99**:421–428.
29. Dekker LR, Coronel R, VanBavel E, Spaan JA, Opthof T. Intracellular Ca^{2+} and delay of ischemia-induced electrical uncoupling in preconditioned rabbit ventricular myocardium. *Cardiovasc Res* 1999;**44**:101–112.
30. Townsend PA, Davidson SM, Clarke SJ, Khaliulin I, Carroll CJ, Scarabelli TM *et al*. Urocortin prevents mitochondrial permeability transition in response to reperfusion injury indirectly by reducing oxidative stress. *Am J Physiol Heart Circ Physiol* 2007; **293**:H928–H938.
31. Baines CP, Song C-X, Zheng Y-T, Wang G-W, Zhang J, Wang O-L *et al*. Protein kinase Cε interacts with and inhibits the permeability transition pore in cardiac mitochondria. *Circ Res* 2003;**92**:873–880.
32. Roy SS, Madesh M, Davies E, Antonsson B, Danial N, Hajnoczky G. Bad targets the permeability transition pore independent of Bax or Bak to switch between Ca^{2+}-dependent cell survival and death. *Mol Cell* 2009;**33**:377–388.
33. Malhotra A, Begley R, Kang BPS, Rana I, Liu J, Yang G *et al*. PKC-ε-dependent survival signals in diabetic hearts. *Am J Physiol Heart Circ Physiol* 2005;**289**:H1343–H1350.
34. Prpic V, Spencer TL, Bygrave FL. Stable enhancement of calcium retention in mitochondria isolated from rat liver after the administration of glucagon to the intact animal. *Biochem J* 1978;**176**:705–714.
35. Taylor WM, Prpic V, Exton JH, Bygrave FL. Stable changes to calcium fluxes in mitochondria isolated from rat livers perfused with alpha-adrenergic agonists and with glucagon. *Biochem J* 1980;**188**:443–450.
36. Hughes BP, Barritt GJ. Effects of glucagon and N6O2′-dibutyryladenosine 3′:5′-cyclic monophosphate on calcium transport in isolated rat liver mitochondria. *Biochem J* 1978;**176**:295–304.
37. Armston AE, Halestrap AP. Glucagon treatment of rats inhibits the accumulation of lysophospholipids by liver mitochondria during preparation and subsequent incubation. *Biosci Rep* 1984;**4**:903–908.
38. Van Kuijk FJGM, Sevanian A, Handelman GJ, Dratz EA. A new role for phospholipase A2: protection of membranes from peroxidation damage. *Trends Biochem Sci* 1987;**12**: 31–34.