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ORIGINAL CONTRIBUTION

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The p38 MAPK inhibitor, SB203580, abrogates ischaemic preconditioning in rat heart but timing of administration is critical

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Abstract There is debate concerning the involvement of p38 mitogen activated protein kinase (MAPK) in the mediation of ischaemic preconditioning. Pharmacological inhibition of p38 MAPK with SB203580 has been reported to block preconditioning in some studies but not in others. We hypothesised that this divergence could be due to differences in the timing of inhibitor administration. Isolated rat hearts were perfused in the Langendorff mode and subjected to 35 min regional ischaemia followed by 120 min reperfusion. Hearts were then double stained with Evans' blue and triphenyltetrazolium chloride to determine risk (R) and infarct zones (I), expressed as I/R % ratios. Preconditioned hearts were subjected to 2 times 5 min global ischaemia with 10 min intervening reperfusion. SB203580 10 μM was perfused either during the preconditioning protocol (PC+SB-early), just prior to and during the first 15 min of the lethal ischaemia (PC+SB-late) or prior to regional ischaemia in the absence of preconditioning. Ischaemic preconditioning significantly limited infarct size (I/R 38.9 ± 3.0 % in control vs 13.4 ± 2.4 %, $P < 0.01$). In the PC+SB-early group, preconditioning was still fully protective (I/R % 14.6 ± 1.0). However, in the PC+SB-late group, SB203580 completely blocked the protection afforded by preconditioning (I/R % 33.6 ± 4.4 %, $P < 0.01$ vs 13.4 ± 2.4 % in preconditioned hearts, $p < 0.05$). SB203580 alone did not affect infarct size when given prior to and during regional ischaemia (I/R 36.2 ± 2.7 %). These histological data are corroborated by a significant increase in p38 MAPK activation in the preconditioned hearts during sustained ischaemia in comparison with the controls. In conclusion the activation of p38 MAPK during lethal ischaemia, but not during the ischaemic preconditioning protocol, is essential for the mediation of protection and may resolve some of the earlier controversy surrounding the use of SB203580 in preconditioning studies.

Key words Ischaemic preconditioning – p38 MAPK – SB203580 – infarct size

Introduction

p38 mitogen activated protein kinase (MAPK), also known as stress-activated protein kinase 2a, is a family of isoenzymes activated in myocardium by oxidative stresses, including ischaemia-reperfusion (4, 6, 12). However, controversy surrounds the role of this kinase in executing pathophysiological responses to ischaemia-reperfusion since the existence of isoforms having different physiological functions confers a complex role of p38 MAPK in the balance between cell survival and cell death (22). Ischaemic preconditioning is the most powerful endogenous mechanism of protection for the infarcted myocardium (3, 11). The involvement of p38 MAPK in the mediation of classical (early) ischaemic preconditioning of myocardium is uncertain (1, 20). Some authors reported that pharmacological inhibition of p38 MAPK blocks precondi-

tioning (17, 18, 20, 23). Also it has been shown in isolated rabbit hearts that anisomycin, a p38 MAPK activator, mimics the anti-infarct effect of ischaemic preconditioning (19). Other studies, in contrast, suggest that p38 MAPK activation is deleterious and that inhibition of p38 MAPK per se is beneficial during sustained ischaemia (2, 5, 15, 16, 24). The divergence in the literature may be explained, at least in part, by issues associated with the timing of enzyme activation and therefore the timing of inhibitor administration in experimental investigations. Thus, if p38 MAPK activation plays a role in eliciting preconditioning protection, it is not clear if activation occurs during preconditioning ischaemia or during the sustained ischaemic insult. Other important concerns may be species differences, inhibitor concentration and model dependency.

Nearly all the reported studies in this area of investigation have employed the p38 MAPK inhibitor, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole]. The aim of the present study was to determine if SB203580 administered at different times in relation to ischaemic preconditioning and sustained ischaemia influenced the anti-infarct response. Our results suggest that p38 MAPK activation during the preconditioning period does not mediate the protective effects of IPC. Rather, in preconditioned hearts p38 MAPK activation is obligatory during the sustained ischaemic period. Our data show that this activation is significantly higher in the preconditioned hearts in comparison with controls. However, in non-preconditioned hearts, inhibition of p38 MAP kinase activity during sustained ischaemia does not confer protection.

Methods

Animals

Male Sprague-Dawley rats (300–350 g body weight) were used. All animals were obtained from the same source, fed standard diet, housed under the same conditions and received humane care in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

Langendorff perfusion

Animals were anaesthetised by IP injection of sodium pentobarbitone (55 mg/kg) and heparin was given simultaneously (300 U). The hearts were excised, placed in ice-cold buffer and within 1 min mounted on a constant pressure Langendorff system (80 mm Hg). They were perfused retrogradely with a modified Krebs-Henseleit (KH) bicarbonate buffer containing (in mM): NaCl 118.5, $NaHCO_3$ 25, KCl 4.8, $MgSO_4$ 1.2, $KH2PO_4$ 1.2, $CaCl_2$ 1.7, glucose 12. All solutions were filtered through a Watman microfibre filter (2.0 μm pore), gassed with 95 %O_2 / 5 % CO_2 and maintained at 37 °C with the help of the water-heated double jacket chambers. The temperature was permanently monitored by a thermocouple inserted in the right ventricle. The pH of the perfusate was checked with the help of a gas analyser, and adjusted if necessary to 7.4 (±0.05) by modifying the gas output. A latex isovolumic balloon was introduced in the left ventricle through an insertion performed in the left atrial appendage and inflated up to 8–10 mm Hg. It was attached by a pressure transducer connected with a Labsystem chart recorder. Left ventricular developed pressure, heart rate and coronary flow were registered at regular intervals. Successful coronary occlusion was confirmed by a reduction in coronary flow rate. Reperfusion was associated with a return of coronary flow rate during the first 10 min after release of the coronary snare.

A surgical needle with 4/0 silk suture was passed under the left main coronary artery and the ends of the thread were passed through a small plastic tube to form a snare. Regional ischaemia was induced by tightening the snare and reperfusion was started by releasing the ends of the thread.

Experimental groups

Hearts were randomly assigned to one of the following experimental protocols. The experimental protocols are presented graphically in Fig. 1. SB203580 (Calbiochem, Nottingham, UK) was dissolved in dimethylsulphoxide (DMSO) and added to the KH buffer to give a final concentration of SB203580 10 μM, and DMSO not more than 0.02 %.

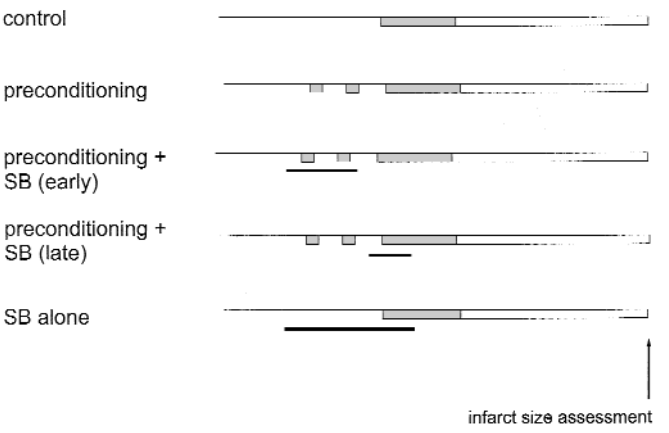

Fig. 1 Experimental protocols. Periods of ischaemia are shaded and periods of reperfusion are unshaded. All hearts were subjected to 35 min regional ischaemia followed by 120 min reperfusion before infarct size assessment. Preconditioning was effected by two 5 min periods of global normothermic ischaemia with 10 min intervening reperfusion. Periods of perfusion with SB203580 10 μM are represented by the black bars.

- *Control*: hearts were allowed to stabilise and were then perfused with 0.02 % DMSO for 20 min during stabilisation, prior to 35 min regional ischaemia and 120 min reperfusion.
- *Preconditioning*: the ischaemic preconditioning protocol consisted of two 5 min periods of global ischaemia with 10 min intervening reperfusion prior of the 35 min lethal injury.
- *Preconditioning + SB(early)*: SB203580 10 μM treatment began 5 min before the first preconditioning cycle and the drug was washed out 5 min prior to the onset of sustained regional ischaemia.
- *Preconditioning + SB(late)*: SB203580 10 μM was commenced 5 min after the preconditioning cycles and continued until 15 min into sustained regional ischaemia.
- *SB alone*: hearts were treated with SB203580 10 μM for 20 during stabilisation, until 15 min after the onset of regional ischaemia.

Infarct size measurement

At the end of 120 min reperfusion, the coronary snare was tightened to reocclude the branch of the coronary artery and a saline solution of 0.12 % Evans' blue was infused slowly into the aorta. This delineated the normally perfused myocardium as a dark blue coloured area, leaving the risk zone unstained. After 1 to 4 hours at –20 °C, hearts were sliced into 1 mm thick transverse sections from apex to base and incubated in triphenyltetrazolium chloride solution (1 % in phosphate buffer, pH 7.4) at 37 °C for 10–12 min. The tissue slices were then fixed in 10 % formalin for not less than 6 h. At the end of this procedure, the viable tissue within the risk zone was stained red and the infarcted tissue appeared pale. The slices were drawn onto acetate sheets. Using a computerised planimetry package (Planimetry Plus version 1.0. Boreal Software, Norway) we calculated the percentage of the infarcted tissue within the volume of the myocardium at risk.

p38 MAPK activation assay

Six control and six preconditioned rat hearts suffered ischaemia during which serial biopsies were taken. Biopsy samples were rapidly frozen (–80 °C) and homogenised using previously reported methods (19). In brief, frozen heart pieces were homogenised with a Polytron tissue homogenizer in cold (4 °C) lysis buffer (75 mM β-glycerol phosphate, 20 mM HEPES, 2 mM EGTA, 2 mM EDTA, 1 mM Na_3VO_4, 0.05 % Triton X-100, 4 mg/ml leupeptin, 1 mM PMSF, pH 7.2) and centrifuged at 13,000 g for 10 min in a cold room. The supernatant was saved for analysis of protein using a modified Lowry method (BioRad, 500-0116), and equal quantities of protein (20 μg) were loaded on 10 % SDS-polyacrylamide gels for electrophoretic separation. Proteins were blotted onto nitro-cellulose and p38 MAPK was probed with phospho-specific antibody. Labelled proteins were visualised by exposure of X-ray film using the chemiluminiscence procedure following manufacturer's instructions (New England Biolabs, Phototype-HRP). The absorbance density of each lane was quantified by densitometry after digital scanning of each film. Densities are normalised as the fraction of the total density within each experiment.

Statistical analysis

All values are expressed as mean ± SE. Infarct size data and the p38 MAPK phosphorylation data were analysed using 1-way analysis of variance and the Fisher's protected least significant difference test for multiple comparisons. Data were considered significant at the $p < 0.05$ level.

Results

Exclusions

Thirty eight hearts were used in total. Four hearts were excluded for the following technical reasons: one heart had a

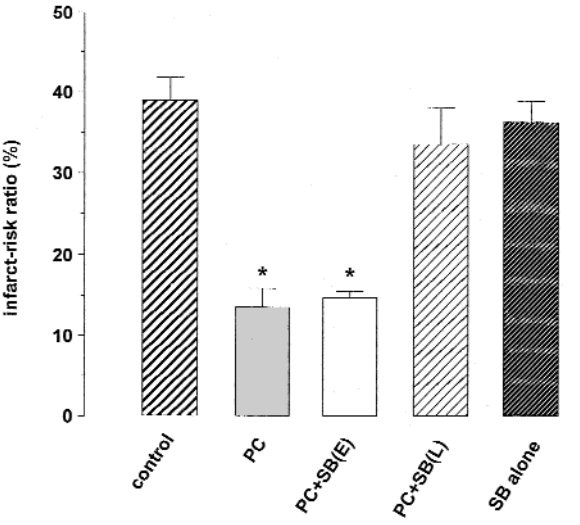

Fig. 2 Infarct size expressed as a percentage of the ischaemic risk zone. Histograms show mean ± SEM. *PC* preconditioning group; *PC+SB(E)* preconditioning + SB (early); *PC+SB(L)* preconditioning + SB (late). * $P < 0.01$ vs control (1-way ANOVA).

Table 1 Body weight, pre-ischaemic contractile function, coronary flow and risk zone volume

Treatment group	n	Body weight (g)	Heart rate (beats/min)	LVDP (mm Hg)	Coronary flow (ml/min)	Risk zone volume (cm^3)
Control	9	350 ± 10	300 ± 5	90 ± 9	10.4 ± 0.5	0.57 ± 0.03
Preconditioning	5	332 ± 8	288 ± 7	88 ± 11	10.6 ± 0.5	0.47 ± 0.03
Preconditioning + SB (early)	7	338 ± 4	291 ± 5	87 ± 8	12.7 ± 1.3	0.52 ± 0.05
Preconditioning + SB (late)	6	340 ± 5	300 ± 12	89 ± 9	11.3 ± 0.5	0.56 ± 0.03
SB alone	7	338 ± 4	285 ± 15	95 ± 3	11.0 ± 1.0	0.53 ± 0.05

LVDP left ventricular developed pressure

very low coronary flow rate at baseline, two hearts had unphysiological left ventricular developed pressure at baseline, one heart failed to stabilise. Thus we report data from 34 successfully completed experiments.

Coronary flow and functional data

Baseline contractile function and coronary flow rates are shown in Table 1. There were no significant differences between groups at the end of the stabilisation period. During regional ischaemia, coronary flow rate declined by approximately 50 % in all groups. Immediately following reperfusion coronary flow recovered and then declined gradually during the following 120 min period. This decline was similar in all experimental groups. Left ventricular developed pressure declined during ischaemia and did not differ at any time point between the experimental groups probably due to a significant degree of myocardial stunning in the viable risk zone. Perfusion with SB203580 10 μM did not influence contractile function or coronary flow rate.

Risk volume and infarct size data

Ischaemic risk zone volume was similar in all experimental groups (Table 1). The primary endpoint of the study, infarct-to-risk ratio (I/R %) is depicted in Fig. 2. Control hearts subjected to 35 min regional ischaemia developed 38.9 ± 3.0 % infarction within the risk zone. Preconditioned hearts developed significantly smaller infarcts in comparison with the control group (I/R 13.4 ± 2.4 %, $P < 0.05$ vs control). When SB203580 was administered during ischaemic preconditioning protocol (Preconditioning + SB (early)), the protective effect of preconditioning was not altered (I/R 14.6 ± 1.0 %, $P < 0.01$ vs control). However when SB203580 was administered after the preconditioning protocol and during early regional ischaemia (Preconditioning + SB (late)), the protection afforded by preconditioning was abolished (I/R 33.6 ± 4.4 %, not significantly different from control). SB203580 prior to regional ischaemia in the absence of preconditioning did not influence infarct size (I/R 36.2 ± 2.7 %, not significantly different from control). These data suggest that p38 MAPK activation during the preconditioning period does not mediate the protective effects of ischaemic preconditioning. Rather, in preconditioned hearts p38 MAPK activation is necessary during the sustained ischaemic period. However, in non-preconditioned hearts, inhibition of p38 MAP kinase activity during sustained ischaemia does not confer protection.

Phosphorylation of p38 MAPK

There was a time-dependent activation of p38 MAPK in the preconditioned hearts (Fig. 3). At 20 min ischaemia the phosphorylation of p38 MAPK increased by more than 200 % over the basal value in comparison with the controls in which this increase did not reach more than 20 %. Analysis of the group

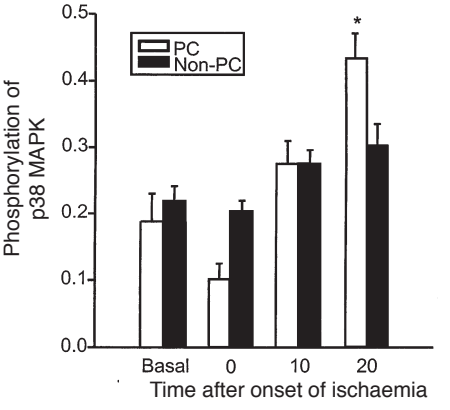

Fig. 3 Effect of preconditioning on p38 MAPK phosphorylation. Biopsies were removed at indicated times in PC (open bars) and control (shaded bars) hearts and p38 MAPK phosphorylation was determined using phospho-specific p38 MAPK antibody (9211S, New England Biolabs). In PC hearts (n = 6), the basal p38 MAPK phosphorylation declined slighty at the onset of index ischaemia (0 min) and then increased at 10 min and increased significantly with time (*) at 20 min of ischaemia ($P < 0.001$ vs basal). By contrast, p38 MAPK phosphorylation remained near basal levels and was not significantly different at all times in control hearts (n = 6).

data from these experiments indicates that the time-dependent increase in the preconditioned group was highly significant ($p < 0.001$), whereas there was no significant change with time in the non-preconditioned group. It is also interesting to note that there was a tendency for the p38 MAPK phosphorylation level to decline at the beginning of the index ischaemia in this group, but the value did not reach significance.

Discussion

The aim of this study was to probe a possible reason for the discordant findings reported with the p38 MAPK inhibitor SB203580 in ischaemic preconditioning studies. We have specifically examined the effects of timing of SB203580 treatment. For this purpose we chose the rat isolated heart model with infarct size as the endpoint. The results indicate that in this experimental model the protection against infarction conferred by ischaemic preconditioning is dependent upon the activation of p38 MAP kinase during sustained ischaemia but not during preconditioning ischaemia. Indeed, our results obtained using the Western blotting technique also show that p38 MAPK activation takes place during index ischaemia at a significantly higher extent in the preconditioned hearts than in controls. This finding concurs with work from Downey and Cohen's laboratory in rabbit myocardium demonstrating that Tyr182 phosphorylation of p38 MAPK is increased during sustained ischaemia following a preconditioning protocol (23). It is also consistent with other studies showing that MAPKAPK2, a kinase distal to p38 MAPK, is increased during lethal ischaemia after a preconditioning protocol (17–19). This implies activation of p38 MAPK, since MAPKAPK2 is immediately downstream. Our finding is also consistent with work by Maulik et al. (18) who showed that SB203580 abolished the protection afforded by preconditioning in isolated working rat heart. However, in that study SB203580 5 μM was perfused for 15 min prior to the preconditioning period and contractile function was employed as the endpoint of protection.

A further finding in the present study is that SB203580 administration prior to and during the first 15 min of sustained ischaemia in non-preconditioned hearts did not exert a protective effect on infarct size. This finding contrasts with several studies undertaken using a variety of experimental models. Barancik et al. (2) reported that local infusion of SB203580 into pig myocardium prior to 60 min coronary artery occlusion resulted in striking limitation of infarct size. However, it is not clear from that study how reliably and predictably tissue concentration of SB203580 could be controlled with such an administration protocol. It is possible that very high concentrations of SB203580 were achieved at certain points in the ischaemic risk zone and that non-specific effects of the agent might influence infarct size under these conditions. Other evidence that p38 MAPK activation during ischaemia is detrimental, or that its inhibition is beneficial, comes from isolated neonatal cardiac myocyte studies (6, 16). Mackay and Mochly-Rosen (16) reported that p38 MAPK was phosphorylated during simulated ischaemia biphasically. Application of SB203580 attenuated cell injury in a concentration-dependent manner and also inhibited the activation of caspase-3. This work suggested a pro-injurious and pro-apoptotic role of p38 MAPK during sustained ischaemia. Further isolated cell studies by Yue et al. (24) also suggest that p38 MAPK is activated during ischaemia and further activated upon reperfusion. The number of cardiac myocytes displaying hallmarks of apoptosis was reduced in the presence of SB242719, a selective p38 MAPK inhibitor, consistent with the notion that p38 MAPK activation during sustained ischaemia and reperfusion is pro-apoptotic. The same group (15) has extended this work to isolated intact rabbit heart and showed that maximal p38 MAPK activation occurred at 10 min reperfusion following 15 min ischaemia. SB203580 10 μM perfused 10 min prior to ischaemia decreased the extent of apoptosis in these hearts and improved post-ischaemic recovery of contractile function and, in separate experiments, limited infarct size. SB203580 was most protective when it was present during both ischaemia *and* reperfusion. Therefore it is possible that p38 MAPK activation is particularly deleterious during reperfusion following sustained ischaemia. In our experimental model, SB203580 was eluted 20 min prior to reperfusion. Finally, Cain et al. (5) have reported that simulated ischaemia in isolated human atrial trabeculae was associated with induction of tumour necrosis factor-α (TNFα). Exposure to SB203580 1 μM for 10 min prior to simulated ischaemia resulted in attenuation of TNFα content and improved recovery of contractile function during reoxygenation. In the study that we report here, contractile function during coronary occlusion and reperfusion did not differ significantly among the groups at any time point during the experimental protocol. It is important to note that contractile function is not a primary endpoint with this model: during the long experimental time course there is considerable rundown in the contractile parameters which preclude detailed interpretation.

SB203580 is described as a potent and specific inhibitor of p38 MAPK with an IC50 of 0.6 mM (8, 12). The agent has been extensively used and is regarded as the classical p38 MAPK inhibitor, blocking the enzyme activity but not its phosphorylation. In consequence, measuring the phosphorylation of downstream substrates, such as MAPKAP kinase 2/3 or hsp 27, not performed for this study, would be necessary to confirm the relevance of p38MAP kinase in preconditioning and it is the subject of ongoing research. The use of dual phospho-specific antibodies is not sufficient to show that SB203580 blocks the activity of p38 MAPK. We have however previously reported, using 2d gel electrophoresis, that the activation of p38 MAPK by the use of an adenosine A1 receptor agonist is followed by the hsp 27 phosphorylation (10). Furthermore,

although originally reported to be without effects on JNK and p42/p44 MAPK activity, several recent reports suggest that SB203580 may not be completely selective for p38 MAPK. For example, Clerk and Sugden (8) have reported that cardiac p38 MAPK is inhibited by SB203580 at an IC50 of 0.07 μM and that JNK (p52/p54 MAPK) is inhibited by the compound at an IC50 in the range 3-10 mM. Lali et al. (13) have shown that in stimulated T-cells, SB203580 inhibits protein kinase B (PKB; Akt) with an IC50 in the range 3–5 μM. Since the majority of studies on myocardial ischaemia-reperfusion using SB203580 have used concentrations of 5 or 10 μM, it is would seem sensible to urge caution in interpreting these studies as evidence of specific p38 MAPK inhibition.

In conclusion these data suggest that SB203580 abrogates the protection afforded by ischaemic preconditioning when the inhibitor is present during the sustained ischaemic period but not during the preconditioning ischaemic period. We found no evidence to support the notion that SB203580 is protective in its own right when administered during the early part of lethal ischaemia although we can not exclude the possibility that the agent may modify injury during reperfusion. Although the concentration of SB203580 used in these studies (10 μM) effectively inhibits p38 MAPK activation, it is possible that other kinases mediating the effects of preconditioning were inhibited by SB203580 since the specificity of this pharmacological tool is questionable. Moreover, it seems that the use of this inhibitor in preconditioning studies may be highly model-dependent, varying not only with the nature of the experimental preparation used and the endpoint selected but also with the timing of inhibitor application.

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