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ORIGINAL CONTRIBUTION

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SB 203580, an inhibitor of p38 MAPK, abolishes infarct-limiting effect of ischemic preconditioning in isolated rabbit hearts

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Abstract There is debate concerning the involvement of p38 mitogen-activated protein kinase (MAPK) in ischemic preconditioning (PC). At the center of the controversy are data obtained after administration of SB 203580, a specific inhibitor of p38 MAPK. Whereas several studies have reported that SB 203580 abolishes the cardioprotective effect of PC, others claim that this compound is actually cardioprotective against ischemia. Many of these latter observations have been made in isolated myocardial cells. Accordingly the present study was designed to test the effect of SB 203580 in a model of preconditioning in intact rabbit hearts in which infarct size was the end-point. Isolated hearts experienced 30 min of regional ischemia followed by 120 min of reperfusion. Infarct size was measured with triphenyltetrazolium chloride. In control hearts infarction was 30.2 ± 3.3 % of the risk zone. PC with 5 min of global ischemia and 10 min of reperfusion before the 30-min period of ischemia significantly reduced infarct size to 10.2 ± 2.4 % ($P < 0.05$ vs. control). SB 203580 (2 μM) added to the perfusate for 20 min starting 5 min before the index ischemia totally blocked the protection from PC (27.4 ± 3.3 % infarction). SB 203580 alone had no effect on infarct size (28.6 ± 4.6 % infarction). These results reveal that SB 203580 does not affect infarct size on its own, but selectively blocks preconditioning's anti-infarct effect in the intact rabbit heart.

Key words Infarction – ischemic preconditioning – p38 MAPK – SB 203580

Introduction

Preconditioning (PC) by brief episodes of ischemia/reperfusion exerts a powerful protective influence on the heart (18). PC is triggered by substances released during short periods of ischemia, including adenosine (10), bradykinin (9), and opioids (25), which are believed to subsequently activate protein kinase C (PKC) during a sustained ischemia (17, 28). However, the cellular mechanism beyond PKC remains a matter of controversy. Recent studies suggest that mitogen-activated protein kinase (MAPK), a family of stress-activated protein kinases, might be involved. One of these, p38 MAPK, has been proposed as the protective kinase that is activated during preconditioning. However, the published observations regarding this kinase are inconsistent. We (26) and others (15) have reported that PC activates p38 MAPK as well as MAPKAPK2 (20), which is the downstream substrate of p38 MAPK. In contrast, other studies have reported that p38 MAPK activation by PC is transient and does not correlate with cardioprotection (1, 22, 24).

At the center of the controversy are data obtained using the specific p38 MAPK inhibitors, SB 203580 and SB 202190. Because of the cost of these compounds few investigators have tested their effects on infarction in intact hearts. In one such study, inhibition of p38 MAPK with SB 203580 completely blocked the anti-infarct effect of PC in isolated rat hearts (16). In support of these observations SB 203580 also blocked protection from PC in a rabbit cardiomyocyte model of simulated ischemia (26). Importantly SB 203580 had no effect on injury in non-preconditioned myocardium and isolated cells. Both of

these observations would indicate that activation of p38 MAPK is an essential step in the PC signaling pathway. In apparent contradiction of these findings, several studies have reported that SB 203580 by itself is quite protective. Improved post-ischemic functional recovery was seen in both isolated rat hearts (23) and in human atrial muscle (5). The drug also reduced infarction in a region where it was directly injected into an in situ pig heart (4), and it delayed cell death in isolated rat cardiomyocytes (14) and myoblasts (19). These observations suggest that p38 MAPK activation is detrimental to the ischemic heart rather than protective. Finally Schneider et al. (24) recently reported that SB 202190, a compound related to SB 203580 with similar p38 MAPK blocking ability, did not affect PC's anti-infarct effect in isolated rat hearts, but did limit infarction in non-PC hearts. Some of these divergent data may be explained, at least in part, by differences in timing of administration of the inhibitor because of the apparent critical dependence of PC on activation of p38 MAPK at a specific point in the signal cascade. More importantly, only two of the above studies actually measured infarct size, the only clinically relevant end-point for an anti-infarct intervention. Accordingly we examined the effect of administration of SB 203580 during the index ischemia on PC's effect in our well-characterized in vitro rabbit heart preparation which uses infarct size as the end-point.

Materials and methods

This study was conducted in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (21).

Surgical preparation

New Zealand White rabbits of either sex were anesthetized with intravenous sodium pentobarbital (30 mg/kg). The trachea was intubated through a cervical incision. Mechanical ventilation was achieved with a positive-pressure respirator (MD Industries, Mobile, AL) using 100 % O_2. A left thoracotomy was performed in the fourth intercostal space and the pericardium opened to expose the heart. A 2-0 silk suture on a curved taper needle was passed around a branch of the left coronary artery and the ends pulled through a small vinyl tube to form a snare. The heart was rapidly excised and mounted on a Langendorff apparatus. The heart was perfused with Krebs-Henseleit bicarbonate buffer containing 118.5 mM NaCl, 4.7 mM KCL, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM glucose. The perfusate was bubbled with a 95 % O_2–5 % CO_2 gas mixture and perfusate temperature was maintained at 38 °C. The coronary perfusion pressure was set at 75 mmHg. A saline-filled latex balloon connected by a catheter to a pressure transducer was inserted into the left ventricle. At the beginning of the experiment, the balloon volume was adjusted to an end-diastolic pressure of 5 mmHg. Atrial pacing was performed at 200 beats/min if the spontaneous rate was lower. Total coronary arterial flow was measured by timed collection of effluent dripping from the heart. The heart was allowed to stabilize for 20 min before the experiment was begun.

Measurement of infarct and risk zones

At the end of each experiment the snare was retightened and 1–10 μm zinc/cadmium sulfide fluorescent particles (Duke Scientific, Palo Alto, CA, USA) were infused into the perfusate to demarcate the risk zone as the tissue with no fluorescence. The heart was then weighed, frozen, and cut into 2-mm thick slices. The slices were thawed and stained by incubation in 1 % triphenyltetrazolium chloride (TTC) in buffer at pH 7.4 for 20 min. After staining, the slices were immersed in 10 % formalin to enhance the contrast between stained and unstained tissue. The areas of infarction (TTC negative) and risk zone (non-fluorescent under ultraviolet light) were determined by planimetry of each slice. Infarct and risk zone volumes were then calculated by multiplying each area by the slice thickness and summing the products. Infarct size was expressed as the percentage of the risk zone infarcted.

Experimental protocols

In all hearts infarction was produced by tightening the snare for 30 min (the index ischemia). Myocardial ischemia was confirmed by a decrease of left ventricular developed pressure (LVDP). The snare was then released and reperfusion permitted for 120 min. Reperfusion was documented by recovery of coronary flow and LVDP. The animals were divided into 4

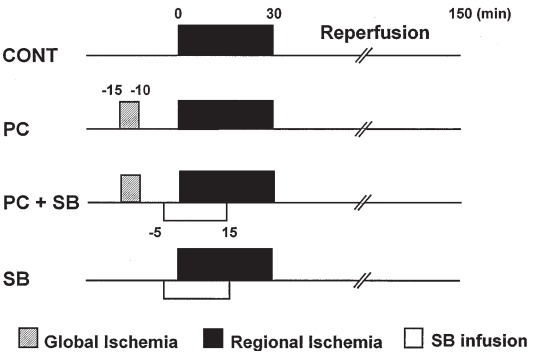

Fig. 1 Experimental protocols in in vitro rabbit hearts. Times are relative to the start of the 30-min period of regional ischemia. *CONT* control, *PC* ischemic preconditioning, *SB* SB 203580

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Table 1 Hemodynamic parameters for in vitro hearts

	Baseline	Pre-ischemia*	Ischemia*	Reperfusion*
HR (bpm)				
Control	206 ± 4	–	200 ± 4	207 ± 7
PC	218 ± 6	208 ± 4	204 ± 3H	203 ± 2H
PC + SB 203580	219 ± 4	225 ± 6	228 ± 8	206 ± 6
SB 203580	219 ± 3	224 ± 7	205 ± 3H	207 ± 4H
LVDP (mmHg)				
Control	116 ± 8	–	45 ± 7H	51 ± 10H
PC	112 ± 7	99 ± 7	72 ± 8H	75 ± 9H
PC + SB 203580	125 ± 2	69 ± 4H	41 ± 5H	78 ± 3H
SB 203580	129 ± 1	82 ± 2H	56 ± 4H	78 ± 4H
CF (ml/min/g)				
Control	7.7 ± 0.5	–	3.6 ± 0.2H	4.5 ± 0.8H
PC	8.9 ± 0.5	9.4 ± 0.3	5.7 ± 0.1H	6.5 ± 0.2H
PC + SB 203580	8.4 ± 0.3	6.7 ± 0.2H	2.8 ± 0.2H	5.1 ± 0.4H
SB 203580	9.0 ± 0.3	6.0 ± 0.6H	3.2 ± 0.4H	4.5 ± 0.3H

Values are mean ± S.E.M. Abbreviations: *CF* coronary flow; *HR* heart rate; *LVDP* developed pressure; *PC* ischemic preconditioning; *Measurements were made just before coronary occlusion (Pre-ischemia), 30 min after coronary occlusion (Ischemia), and 30 min after reperfusion (Reperfusion). HP< 0.05 vs. Baseline

groups (Fig. 1). The control group experienced only 30 min of ischemia and 120 min of reperfusion. Hearts in the ischemic PC group were subjected to 5 min of global ischemia followed by 10 min of reperfusion prior to the index ischemia. In the PC + SB group, infusion of SB 203580 (2 μM), a potent p38 MAPK inhibitor, was commenced after preconditioning and 5 min prior to the index ischemia and continued for 20 min. In the SB group, non-preconditioned hearts were treated with 20 min of SB 203580 commencing 5 min prior to ischemia.

Chemicals

SB 203580 was purchased from Sigma Chemicals (St. Louis, MO, USA). It was dissolved in DMSO and finally diluted in Krebs buffer immediately prior to the experiments.

Statistics

All values are given as means ± S.E.M. Changes in hemodynamics were evaluated by analysis of variance with replication. One-way ANOVA combined with Scheffé's post hoc test was used to test for differences in infarct size and baseline hemodynamics between groups. A value of $P < 0.05$ was considered significant.

Results

Table 1 presents the hemodynamic data. There were no significant differences in baseline values for heart rate, left ventricular developed pressure or coronary flow in any of the experimental groups. Infusion of SB 203580 significantly decreased developed pressure and coronary flow. In all groups, both developed pressure and coronary flow were significantly diminished following 30 min of coronary occlusion, with partial recovery observed during the first 30 min of reperfusion. Although there was a trend for left ventricular developed pres-

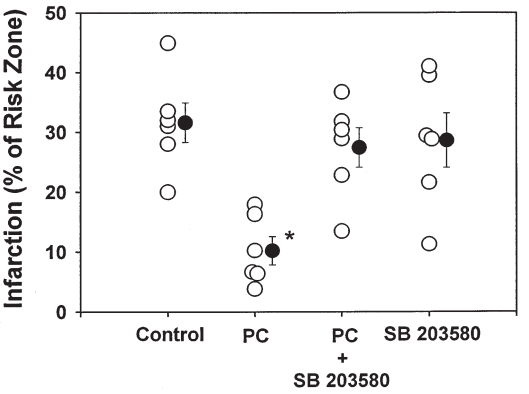

Fig. 2 Graph of the effect of ischemic preconditioning (PC) and SB 203580 on infarct size measured as a percentage of risk zone in in vitro hearts. The protective effect of PC was completely blocked by SB 203580. SB 203580 alone had no effect on infarct size. Open and closed circles represent individual infarct sizes and group means ± S.E.M., respectively. *P< 0.05

Table 2 Infarct size data for PC and SB 203580-treated hearts

	N	Body weight (kg)	Heart weight (g)	Risk zone (cm^3)	Infarct (cm^3)	Infarct size % of risk zone
Control	6	2.1 ± 0.1	6.8 ± 0.2	0.85 ± 0.14	0.28 ± 0.06	32.0 ± 3.3
PC	6	2.1 ± 0.1	6.8 ± 0.2	1.04 ± 0.12	0.11 ± 0.03*	10.2 ± 2.4 *
PC + SB 203580	6	2.0 ± 0.1	6.1 ± 0.1	0.91 ± 0.09	0.25 ± 0.04	27.4 ± 3.3
SB 203580	6	2.0 ± 0.1	6.1 ± 0.1	0.90 ± 0.09	0.27 ± 0.05	28.6 ± 4.6

Values are mean ± S.E.M. Abbreviations: see Table 1; *N* number of animals; *P < 0.05 vs. Control

sure to be higher during ischemia in the PC rabbits and those treated with SB 203580 prior to ischemia, 2-way ANOVA with replication demonstrated no significant group effect among the 4 groups of rabbits.

Table 2 presents group infarct size data, while Fig. 2 graphically depicts infarct size as a percentage of risk zone in individual hearts. There were no significant differences in body weight, heart weight, or risk zone size in any of the experimental groups. In control rabbits, infarct size averaged 32.0 ± 3.3 % of the risk zone. As expected, ischemic PC significantly reduced the amount of infarction to 10.2 ± 2.4 % ($P < 0.05$ vs. control). When infusion of SB 203580 was started after the PC protocol and continued during early regional ischemia, the protection by PC was completely abolished (27.4 ± 3.3 %). Importantly, SB 203580 alone did not influence infarct size (28.6 ± 4.6 %).

Discussion

We have specifically examined the effects of SB 203580 treatment on PC. For this purpose we chose the isolated rabbit heart with infarct size as an end-point. The results indicate that in this experimental model PC's protection against infarction was abolished by SB 203580 when this drug was administered just prior to and during the initial 15 min of the index ischemia. SB 203580 alone did not modify infarct size in non-preconditioned hearts. These results further support the contention that activation of p38 MAPK is required to elicit cardioprotection in the preconditioned heart.

SB 203580 has been extensively studied and is regarded as the classical p38 MAPK inhibitor (IC_{50} = 0.07 μM). In this study, we did not directly demonstrate whether 2 μM SB 203580 is an appropriate dose to inhibit p38 MAPK, although we have previously shown that 10 μM SB 203580 totally abolishes the activation of p38 MAPK's downstream substrate, MAPKAPK2, by PC in the isolated rabbit heart (20). We lowered our dose for the current study because Clerk and Sugden (6) reported that SB 203580 at 10 μM can also inhibit JNK (c-Jun kinase). While they suggested that a dose of 1 μM is selective for p38 MAPK, Ma et al. (12) reported that in rabbit hearts a dose of 1 μM was only partially effective at blocking p38 MAPK. Because Clerk and Sugden (6) found that SB 203580 at 2 μM blocked phosphorylation of MAPKAPK2 in the intact rat heart, indicating an effective blockade of p38 MAPK, we settled on that dose.

Our data cannot support the proposal that SB 203580 alone has any cardioprotective effect against infarction. Several studies have concluded that activation of p38 MAPK is detrimental to the ischemic heart. Inhibition of p38 MAPK by SB 203580 caused a dose-dependent enhancement of cell viability and reduced levels of caspase-3 during prolonged ischemia in cultured neonatal rat cardiomyocytes (13). Cell viability was also enhanced in response to lethal ischemia when p38 MAPK was blocked with this drug in a similar neonatal rat myoblast model (19). We have no clear explanation for differences between our and these other data, but it is likely that these cell models do not exactly mimic the dynamics of infarction in a whole heart.

SB 203580 has also been tested in several infarct size models. Maulik et al. (16) tested the compound in an isolated rat heat and their results were similar to the present findings. However, Ma et al. (12) noted that SB 203580 administered before global ischemia and continuing in reperfusion improved post-ischemic functional recovery, attenuated evidence of apoptosis, and diminished infarct size. More recently Schneider et al. (23) found that a similar drug, SB 202190, had no effect against PC's anti-infarct effect and protected non-preconditioned hearts. Finally, Barancik et al. (4) reported that local infusion of SB 203580 into pig myocardium prior to 60 min of ischemia also resulted in limitation of infarction. The latter model suffers from an inability to control local drug concentration so a non-specific effect cannot be excluded. Unfortunately, there is no easy explanation for the opposing conclusions of the other two studies which used almost identical models.

Our previous reports (11, 27) indicated that the mechanism of preconditioning involves two phases: a trigger phase characterized by a phosphorylation-independent process and a mediation phase occurring during the prolonged ischemic period and requiring phosphorylation of kinases. In this study, we did not show whether SB 203580 could block the trigger phase of the PC protocol. However, it is apparent from the data that activation of p38 MAPK is required during the sustained ischemia to elicit a PC effect. Our previous report had also shown that activation of p38 MAPK in the preconditioned heart was observed only during the mediation, and not during the trigger, phase (26).

There is considerable evidence that activation of p38 MAPK is an important step in preconditioning. We previously reported SB 203580 blocked preconditioning's protection against osmotic fragility in rabbit cardiomyocytes during simulated ischemia (26), while Maulik et al. (16) demonstrated that this drug prevented preconditioning's favorable effects on post-ischemic left ventricular function in intact rat hearts. Furthermore, anisomycin, an activator of both p38 MAPK and JNK, can mimic preconditioning's infarct-limiting effect in rabbit (2) and pig (3) hearts. In a recent study we also measured the activity of MAPKAPK2, a substrate kinase immediately downstream of p38 MAPK in the signaling pathway (20). These results again revealed that this kinase was activated only during ischemia, and then only if the heart had been previously preconditioned. Additionally ischemic preconditioning in the rat heart results in phosphorylation and translocation of constitutive low molecular weight stress proteins (8), particularly αB–crystalline and HSP27, end-products of activa-

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tion of p38 MAPK, while delayed preconditioning in the rabbit results in an increase in phosphorylated isoforms of HSP27 (7). These data imply that p38 MAPK is part of the cardioprotective pathway.

In summary, this test of SB 203580, a p38 MAPK inhibitor, in whole rabbit hearts reveals that the drug blocks the anti-infarct effect of ischemic preconditioning. Because the block occurred when this drug was present during only the sustained ischemia, we conclude that p38 acts during the mediator phase of preconditioning. Thus, activation of p38 MAPK is an essential step in mediating preconditioning in the rabbit heart.

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