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Nuclear factor- κ B inhibition provides additional protection against ischaemia/reperfusion injury in delayed sevoflurane preconditioning

Mojca Remskar Konja^a, Saul Schaefer^b and Hong Liu^a

Background and objective Sevoflurane anaesthetic preconditioning (SPC) has been shown to limit nuclear factor- κ B (NF- κ B) activation and the production of inflammatory cytokines during myocardial ischaemia/reperfusion (I/R). Similarly, pharmacological inhibition of NF- κ B using parthenolide is effective in limiting I/R injury. We, therefore, postulated that the protective effect of delayed SPC would be enhanced by pharmacological NF- κ B inhibition during I/R.

Methods Hearts from 2-month-old male Fisher 344 rats were exposed to 25 min global ischaemia followed by 60 min reperfusion. Rats were divided into four groups prior to I/R: control group; parthenolide group, treated with the I κ B kinase inhibitor parthenolide intraperitoneally 10 min prior to heart isolation; SPC group, treated for 60 min with sevoflurane 48 h prior to heart isolation; and SPC + parthenolide group, treated with SPC for 1 h followed by parthenolide 48 h later. Infarct area, left ventricular function and Ca^{2+} , were measured after I/R.

Results Delayed SPC + parthenolide resulted in greater protection than either intervention alone, resulting in a significant reduction in infarct area and left ventricular

developed pressure (mmHg; 84 ± 19 compared with 15 ± 14 in control hearts; $P = 0.007$). Left ventricular end-diastolic pressure also remained close to baseline values (9 ± 2 mmHg, $P = 0.02$) during I/R, and the increase in Ca^{2+} , seen with I/R was significantly blunted ($P = 0.005$).

Conclusion SPC followed by parthenolide provides a significant protection from I/R injury in this model. As each intervention alone limits NF- κ B activation with I/R, these data are consistent with additive effects of these dual modalities in limiting I/R injury due to NF- κ B activation. *Eur J Anaesthesiol* 26:496–503 © 2009 European Society of Anaesthesiology.

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Keywords: delayed preconditioning, myocardium, nuclear factor- κ B, rat, sevoflurane

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Introduction

Myocardial ischaemia/reperfusion (I/R) results in cellular and mitochondrial accumulation of Ca^{2+} , activation of protein kinases, phospholipases, inducible nitric oxide synthase and expression of preformed adhesion molecules [1–3]. This is followed by activation of nuclear factor- κ B (NF- κ B) that leads to synthesis of inflammatory mediators such as tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1) [4–9]. Early sevoflurane anaesthetic preconditioning (SPC), defined as brief exposure to the inhalational anaesthetic sevoflurane immediately prior to I/R, is protective against myocardial I/R showing limitation of infarct size and/or reduction in intracellular and mitochondrial Ca^{2+} accumulation [10] and lower levels of inflammatory cytokines and NF- κ B activation on reperfusion [7]. Although the mechanism of SPC protection is unknown, studies of ischaemic and morphine preconditioning suggest that SPC may result in reactive oxygen species (ROS)-mediated activation of NF- κ B prior to I/R, with consequent expression of proteins that are protective following I/R [11,12]. These proteins include I κ B α , which would limit NF- κ B activation and cytokine production on I/R through a well described feedback mech-

anism [13], and cytoprotective proteins such as Bcl-2, which would limit mitochondrial death and cellular apoptosis [14,15].

Delayed anaesthetic preconditioning, defined as an I/R injury limiting effect 24–72 h after the initial anaesthetic exposure, has been demonstrated in multiple models [16,17] and appears to share many of the mechanisms of early anaesthetic preconditioning [18,19]. Although not demonstrated with anaesthetic preconditioning, other models of preconditioning have shown increased activation of NF- κ B by a preconditioning stimulus [12,20–22] and decreased NF- κ B activity following renal and cardiac I/R [23–26].

As with SPC alone, pharmacological inhibition of NF- κ B with a number of different NF- κ B inhibitors, including the sesquiterpene lactone parthenolide (parth), has also been demonstrated to protect the myocardium against I/R injury associated with inhibition of I κ B kinase (IKK) activity [27], enhanced stability of I κ B α and inhibition of nuclear translocation of NF- κ B [28]. As both SPC and parth probably protect by limiting NF- κ B activation on

reperfusion, we hypothesized that dual inhibition of NF- κ B by delayed SPC followed by parth would provide greater protection against I/R injury than either delayed SPC or parth alone.

Methods

The present study was approved by the Animal Care Committee of the University of California Davis (Davis, California), and all experiments were conducted in accordance with guidelines of animal care from the National Institutes of Health and the American Physiological Society.

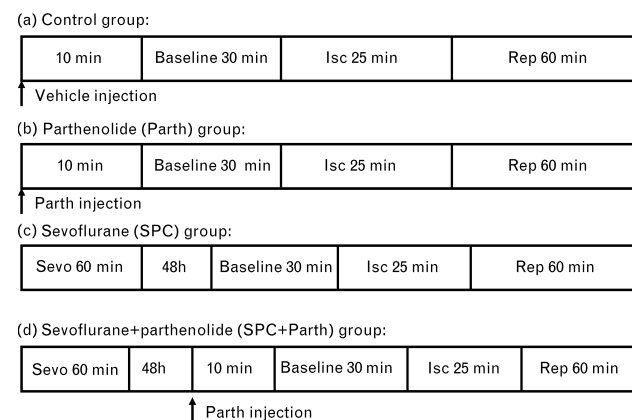
Ischaemia/reperfusion protocol

Two-month-old male Fisher 344 rats were anaesthetized with intraperitoneal (i.p.) pentobarbital (65 mg kg^{-1}) and anticoagulated with i.p. heparin (1000 U kg^{-1}) [16,29]. Rat hearts were harvested and immediately perfused using a Langendorff perfusion system at a pressure of $140 \pm 20 \text{ cmH}_2\text{O}$ at 37°C with modified Krebs–Henseleit bicarbonate solution equilibrated with humidified 95% $\text{O}_2/5\% \text{ CO}_2$ to produce a pH of 7.40 ± 0.05 . The perfusate consisted of $127 \text{ mmol l}^{-1} \text{ NaCl}$, $4.75 \text{ mmol l}^{-1} \text{ KCl}$, $1.25 \text{ mmol l}^{-1} \text{ MgCl}_2$, $2.5 \text{ mmol l}^{-1} \text{ CaCl}_2$, $2.5 \text{ mmol l}^{-1} \text{ NaHCO}_3$ and $10 \text{ mmol l}^{-1} \text{ glucose}$. A catheter with a balloon filled with water was introduced into the left ventricle (LV) through the left atrial appendage. Left ventricular end-diastolic pressure (LVEDP) was set at between 5 and 10 mmHg. Atrial pacing at 5 Hz was maintained during all phases of experiment except during global ischaemia time. Following 30 min of equilibration, the hearts underwent global ischaemia for 25 min, followed by 60 min of reperfusion. At the end of this I/R protocol, the hearts were then sectioned into 2 mm slices for staining for infarct size.

Experimental design

Forty-eight rats were used in this study (the Ca^{2+} experiments were done separately from the haemodynamic experiments). Four groups of six rats in each group were randomly assigned to the following protocols prior to I/R (Fig. 1). In the control group (a), rats were injected i.p. with vehicle ($0.05\% \text{ Tween } 80$, 1 ml kg^{-1}) 10 min before the I/R protocol. In the parth group (b), the NF- κ B inhibitor parth $125 \mu\text{g kg}^{-1}$ was injected i.p. 10 min before the I/R protocol. In the SPC group (c), the rats were exposed to 2.5% sevoflurane delivered with a Sevotec5 variable bypass vaporizer (Datex-Ohmeda, Milwaukee, Wisconsin, USA) with $95\% \text{ O}_2$ and $5\% \text{ CO}_2$ for 1 h in a chamber equipped with monitoring for sevoflurane, O_2 and CO_2 . After sevoflurane exposure, the rats were recovered and returned to their cages for 48 h. At the end of the 48 h period, the hearts were harvested and underwent the I/R protocol. In the SPC + parth group (d), rats were exposed to 2.5% sevoflurane for 1 h, and then left to recover for 48 h. They were then injected with parth, $125 \mu\text{g kg}^{-1}$ i.p., followed by the I/R protocol

Fig. 1



Study design. Rats were divided into four groups. (a) Control group treated with intraperitoneal injection of vehicle 10 min prior to heart isolation. (b) Parthenolide (parth) group treated with intraperitoneal injection of $125 \mu\text{g kg}^{-1}$ sesquiterpene lactone parth 10 min prior to heart isolation. (c) Sevoflurane (SPC) group treated with sevoflurane (sevo) for 1 h, 48 h prior to heart isolation. (d) Sevo + parth (SPC + parth) group treated with sevo for 1 h followed 48 h later by parth injection. Hearts were harvested 10 min after parth injection. After isolation all hearts were immediately Langendorff perfused, equilibrated for 30 min, exposed to global ischaemia (isc) for 25 min and followed by reperfusion (rep) for 60 min.

10 min later. The respiratory rate, O_2 concentration and CO_2 were monitored during sevoflurane exposure, whereas delivered and exhaled gas concentration was monitored with an Ohmeda Rascal II anaesthetic monitor (Ohmeda, Salt Lake City, Utah, USA) [16].

Haemodynamic measurements

Left ventricular pressures were measured with the LV latex balloon filled with water and connected to a pressure transducer (Medex, Dublin, California, USA). Pressures were recorded using Powerlab 4/20 hardware with an amplifier (ADInstruments, Colorado Springs, Colorado, USA) and Chart for Windows version 4.04 software (ADInstruments). The parameters followed to evaluate LV performance were LV developed pressure (LVDP: calculated as LV systolic pressure minus LVEDP), $\text{dp/dt}_{\text{max}}$ as a measure of contractility, $\text{dp/dt}_{\text{min}}$ as a measure of relaxation and LVEDP as a measure of diastolic function.

Myocardial infarct size

Myocardial injury was evaluated by measuring infarct area. At the end of reperfusion, hearts were sliced into 2 mm sections. They were immersed into buffered 2% 2,3,5-triphenyltetrazolium chloride (TTC) with pH adjusted to 7.40 and incubated for 15 min at 37°C . After incubation, the slices were rinsed with H_2O and incubated for 24 h in 4% formaldehyde. Slices were then scanned into the computer using Adobe Photoshop software (Adobe, San Jose, California, USA). NIH image 1.62 (National Institute of Health, Bethesda, Maryland, USA) was used to measure the area of infarction. Infarcted

myocardium is unable to reduce TTC and appears pale compared with viable myocardium that is red secondary to the reduction of TTC. The total areas of infarction were divided by total area of myocardium to determine the percentage area of infarction [16,30,31].

Intracellular calcium: NMR spectroscopy

^{19}F NMR spectroscopy

To measure $[\text{Ca}^{2+}]_i$, hearts from four different groups of animals undergoing the same protocol as above ($n=6$ in each group) were loaded for 30 min with perfusate containing the acetoxymethyl ester 5F-1, 2-bis (2-aminophenoxy)ethane- N,N,N,N -tetraacetic acid (5F-BAPTA) at $2.5 \mu\text{mol l}^{-1}$. FBAPTA was washed out of the extracellular space with control solution before measurement of $[\text{Ca}^{2+}]_i$. ^{19}F spectra were generated from the summed free induction decays of 1500, 45° excitation pulses using 2K word data files and ± 5000 Hz sweep width. $[\text{Ca}^{2+}]_i$ was calculated by using the following formula: $[\text{Ca}^{2+}]_i = K_d [\text{bound}]/[\text{free}]$, where the ratio of Ca^{2+} -bound and free FBAPTA concentrations was equal to the ratio of their corresponding peak areas and K_d is 300 nmol l^{-1} in our experiment [16,30,31].

Data analysis

Data are presented as means $\pm$ SD. Analysis of variance (ANOVA) for repeated measurements was used to test the difference between groups. Two-tailed Student–

Neuman–Keuls posttest was used if the ANOVA was significant. A value of P less than 0.05 was considered statistically significant. Each of the haemodynamic datum point in the figures is at the end of a 10 min interval. Each of the Ca datum points is a summary of a 5 min interval.

Results

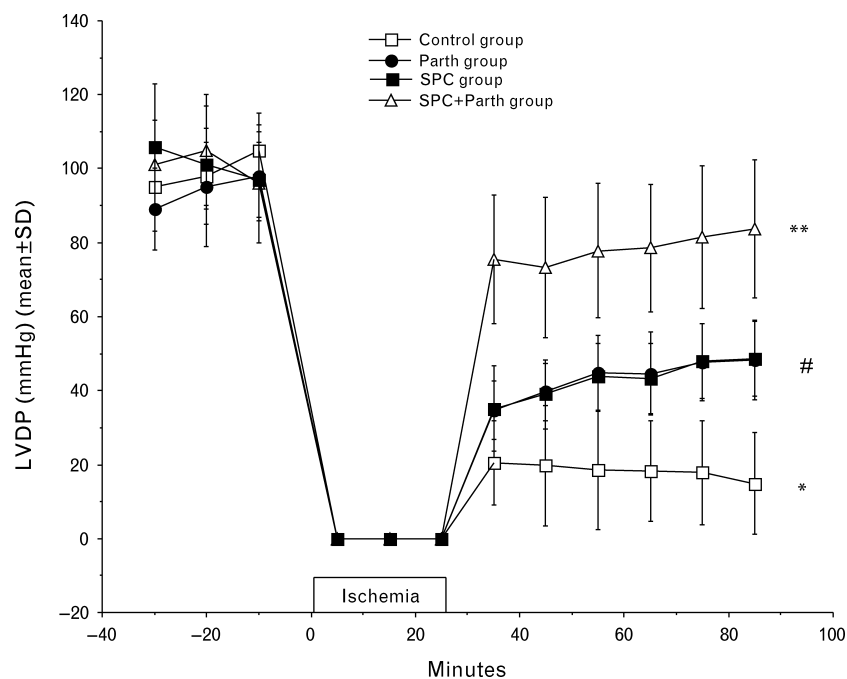
Infarct area

All treatment groups had statistically significantly decreased infarct size as a percentage of total myocardium compared with the control group (parth: $18 \pm 6\%$, SPC: $19 \pm 6\%$, SPC + parth: $10 \pm 4\%$ vs. control: $59 \pm 6\%$; $P=0.023$). The SPC + parth group had further reduction in infarct size when compared with parth or SPC treatment alone ($P=0.034$).

Left ventricular systolic and diastolic function

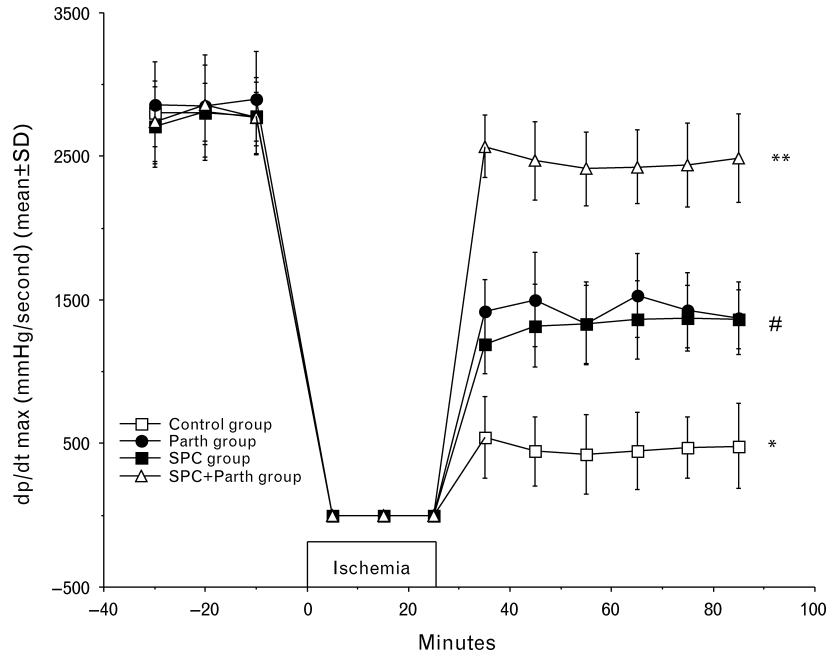
The recovery of LV performance, as quantified during the 60 min reperfusion period vs. the baseline phase prior to ischaemia, is summarized in Figs 2–4. The recovery of LVDP (mmHg) in the parth and SPC groups was significantly greater than in the control group (48 ± 11 and 49 ± 10 vs. 15 ± 14 , respectively; $P=0.02$) at the end of reperfusion. The SPC + parth group had the best recovery compared with the control group (84 ± 19 vs. 15 ± 14 , $P=0.007$). As with infarct size, the SPC + parth group had greater recovery than either treatment alone ($P=0.03$, Fig. 2).

Fig. 2



Left ventricular developed pressure (LVDP) (mmHg) is presented during the whole experiments for control, parthenolide (parth), sevoflurane (SPC) and sevoflurane + parthenolide (SPC + parth) groups. $N=6$ for each group. Means $\pm$ SD for each group. The statistical analysis was performed during reperfusion. * $P=0.02$, control vs. parth and SPC. # $P=0.03$, parth and SPC vs. SPC + parth. ** $P=0.007$, SPC + parth vs. control.

Fig. 3

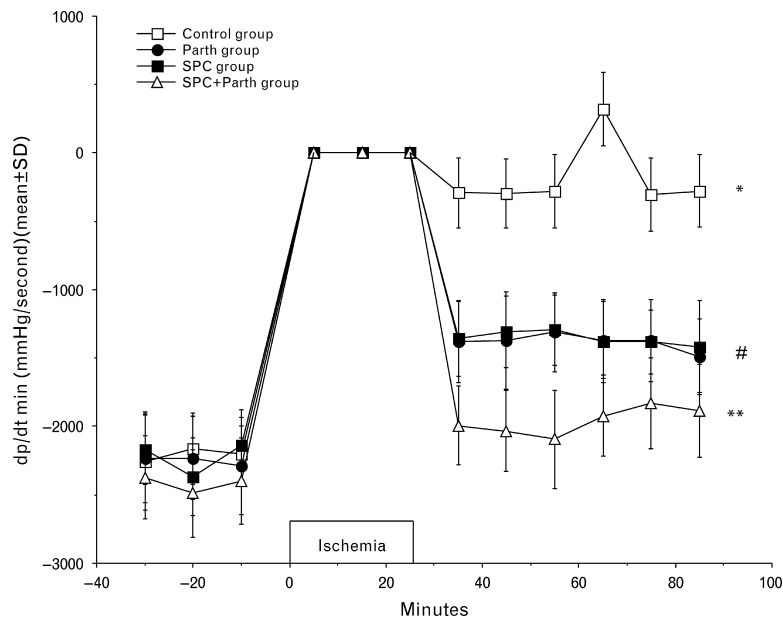


The $dp/dt_{\max}$ (mmHg s^{-1}) is presented throughout the experiments for control, parthenolide (parth), sevoflurane (SPC) and sevoflurane + parthenolide (SPC + parth) groups. $N=6$ for each group. Means $\pm$ SD for each group. The statistical analysis was performed during reperfusion. * $P=0.02$, control vs. parth and SPC. # $P=0.024$, parth and SPC vs. SPC + parth. ** $P=0.007$, SPC + parth vs. control.

Similar findings were observed for $dp/dt_{\max}$ (Fig. 3), which can be used as a measure of LV contractility, and $dp/dt_{\min}$ (Fig. 4), which can be used as a measure of LV relaxation. $dp/dt_{\max}$ (mmHg s^{-1}) in the

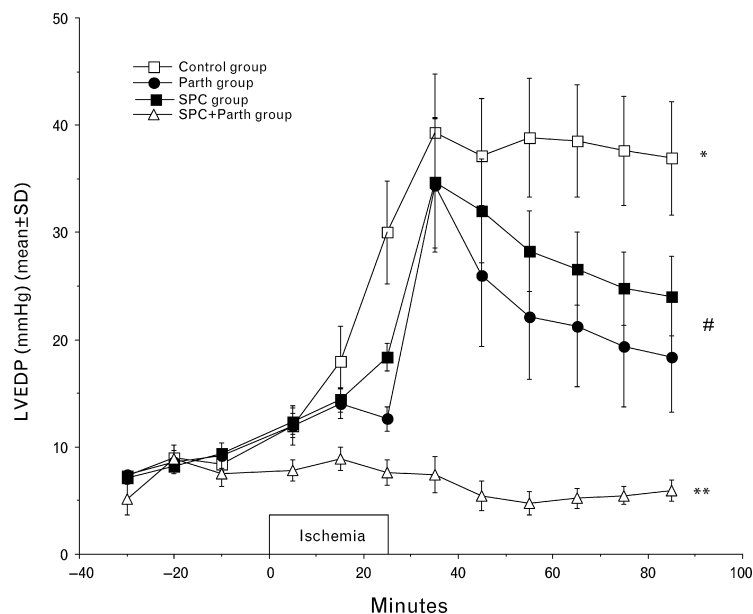
SPC + parth group had significantly greater recovery after I/R, compared with the parth or SPC groups alone (2486 ± 307 vs. 1374 ± 256 , 1368 ± 204 , respectively; $P=0.024$). All groups were statistically different from

Fig. 4



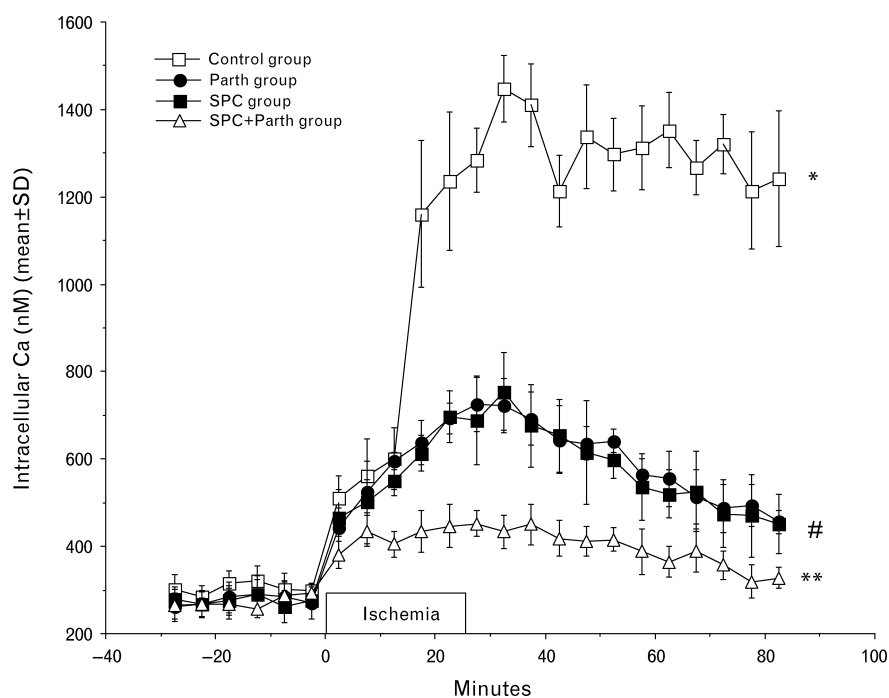
The $dp/dt_{\min}$ (mmHg s^{-1}) is presented during the experiments for control, parthenolide (parth), sevoflurane (SPC) and sevoflurane + parthenolide (SPC + parth) groups. $N=6$ for each group. Means $\pm$ SD for each group. The statistical analysis was performed during reperfusion. * $P=0.04$, control vs. parth and SPC. # $P=0.03$, parth and SPC vs. SPC + parth. ** $P=0.007$, SPC + parth vs. control.

Fig. 5



The left ventricular end-diastolic pressure (LVEDP) (mmHg) is presented during baseline, ischaemia and reperfusion periods for the control, parthenolide (parth), sevoflurane (SPC) and sevoflurane + parthenolide (SPC + parth) groups. $N=6$ for each group. Means $\pm$ SD for each group. The statistical analysis was performed during reperfusion. * $P=0.042$, control vs. parth and SPC. # $P=0.035$, parth and SPC vs. SPC + parth. ** $P=0.02$, SPC + parth vs. control.

Fig. 6



Intracellular Ca^{2+} (nmol l^{-1}) was measured before, during and after ischaemia in the control, parthenolide (parth), sevoflurane (SPC), and sevoflurane + parthenolide (SPC + parth) groups. The SPC + parth group has the smallest increase among all the groups at the end of ischaemia and reperfusion. $N=6$ for each group. Means $\pm$ SD for each group. The statistical analysis was performed during reperfusion. * $P=0.02$, control vs. parth and SPC. # $P=0.014$, parth and SPC vs. SPC + parth. ** $P=0.005$, SPC + parth vs. control.

the control group (484 ± 299 ; $P=0.02$). Among all the treatment groups, the SPC + parth group had the most significant recovery of $dp/dt_{\max}$ compared with the control group ($P=0.007$). In Fig. 4, the preservations in $dp/dt_{\min}$ (mmHg s^{-1}) were significantly better in the parth and SPC groups (-1491 ± 279 and -1416 ± 334 , respectively) than in the control group (-278 ± 178 , $P=0.04$). The SPC + parth group had a better recovery of $dp/dt_{\min}$ (-1886 ± 342) than other treatment groups ($P=0.03$). In the SPC + parth group, the $dp/dt_{\min}$ was best preserved compared with the control group ($P=0.007$).

The development of diastolic dysfunction monitored by LVEDP was significantly lower in the SPC + parth group than in the control (9 ± 2 vs. 37 ± 13 mmHg, $P=0.02$), parth and SPC groups (9 ± 2 vs. 18 ± 5 , 24 ± 9 mmHg, respectively; $P=0.035$) as shown in Fig. 5. The LVEDP in the parth and SPC groups also had a better recovery than in the control group ($P=0.042$).

Intracellular Ca^{2+}

I/R injury caused an increase in intracellular Ca^{2+} (nmol l^{-1}). In the control group, Ca^{2+} increased from a baseline of 300 ± 35 to 1240 ± 154 nmol l^{-1} at the end of reperfusion ($P=0.003$, Fig. 6). Compared with the control group, significant and similar reductions in Ca^{2+} accumulation were seen in the parth and SPC groups (455 ± 25 and 450 ± 68 nmol l^{-1} , respectively; $P=0.02$) at the end of reperfusion. The SPC + parth group had a significant reduction in Ca^{2+} accumulation compared with either treatment group alone ($P=0.014$). Notably, the SPC + parth animals showed the most significant reduction in Ca^{2+} accumulation (328 ± 25 nmol l^{-1} , $P=0.005$) at the end of reperfusion compared with the control group.

Discussion

The present study demonstrates that delayed SPC followed by pharmacological inhibition of NF- κ B with parth 10 min prior to I/R provides significant and additive protection in rat hearts from I/R injury as measured by indices of infarct size, left ventricular systolic and diastolic function and intracellular Ca^{2+} .

Prior studies [5–7,32] have indicated that the reduction in I/R injury in early SPC, defined as I/R occurring within minutes following sevoflurane preconditioning, is associated with attenuation of NF- κ B activation on reperfusion and a reduced inflammatory response. Delayed SPC, defined as I/R occurring 24–72 h after anaesthetic exposure, appears to have common elements with early SPC [10,16,18,33–35]. Although a reduction in NF- κ B activation on reperfusion following ischaemia has not been demonstrated with delayed SPC, it has been shown in other models of preconditioning [23–26].

Pharmacological inhibition of NF- κ B activation with parth and other agents has been shown to decrease I/R

injury in several models in the absence of a preconditioning stimulus [28,36]. In the current experiments, the independent protective effects of both delayed SPC and pharmacological NF- κ B inhibition prior to I/R were confirmed. Each intervention alone had significant beneficial effects on infarct size, left ventricular systolic and diastolic function and calcium accumulation following I/R.

In addition to individual benefit, these experiments showed that delayed SPC and pharmacological inhibition of NF- κ B immediately prior to I/R had additive effects on these endpoints, and provided almost total protection against I/R injury. There may be several reasons for this additive effect. One possibility is that delayed SPC caused reduced NF- κ B activation on reperfusion, as seen with early SPC [7] and other delayed preconditioning models [23–26], and that the additional inhibitory effect of parth on NF- κ B activation with I/R resulted in an even lower NF- κ B activation and resultant reduction in cytokine production [7,10]. Another possibility is that delayed SPC had protective effects via other mechanisms (e.g. Bcl-2 expression, mitoK_{ATP} channel opening) [10,14,37–39], and that parth had the complementary effect of NF- κ B inhibition.

In addition to salutary effects on function and infarct size, delayed SPC and parth had individual and additive effects on intracellular Ca^{2+} overload with I/R. Calcium overload is thought to be a cause of an increase in cellular metabolism in an oxygen-depleted environment, as well as mitochondrial disruption and permeability transition pore opening [3]. Opening of sarcolemmal and mitochondrial K_{ATP} (mitoK_{ATP}) channels by preconditioning prevents Ca^{2+} influx and, thereby, reduces Ca^{2+} loading [40–42]. Early SPC has been shown to reduce Ca^{2+} accumulation on I/R, an effect dependent on mitoK_{ATP} channels [10,33]. In the current experiments, pharmacological inhibition of NF- κ B immediately prior to I/R reduced Ca^{2+} accumulation to a similar degree to delayed SPC. This effect may be due to the previously demonstrated role that NF- κ B plays in activation and perpetuation of the cellular inflammatory response through stimulation of synthesis of factors such as IL-1 and TNF- α [7].

Our study has several potential limitations. We did not specifically investigate different mechanisms involved in delayed SPC and pharmacological NF- κ B inhibition, and did not measure the degree of NF- κ B activation on reperfusion. However, prior studies have shown reduced NF- κ B activation with I/R with both early SPC and pharmacological NF- κ B inhibition alone [7,39]. In addition, we did not investigate the pharmacokinetics and pharmacodynamics of parth, basing the dosing and timing on previous studies [27,28]. As parth was given immediately before I/R, and on the basis of the results of

prior studies and the results of our experiments, we believe that the effect of parth was both lasting and substantial [43]. Although parth is recognized as an I κ B kinase complex inhibitor [28], recent studies [44,45] suggest that parth could affect other pathways without inhibiting NF- κ B. Finally, another control group, that of SPC + vehicle given 48 h prior to I/R, was not included as the vehicle used in this study has not shown any protective effects in prior studies [28,46].

In conclusion, our study demonstrates that combined treatment with delayed SPC followed by the NF- κ B inhibitor parth significantly decreases infarct size, improves LV function and decreases intracellular Ca²⁺ overload with I/R compared with either intervention alone. These data show that targeting pathways of NF- κ B activation using complementary modalities can be an effective method of limiting I/R injury.

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