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Activation of Akt and cardioprotection against reperfusion injury are maximal with only five minutes of sevoflurane postconditioning in isolated rat hearts*

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Abstract: It had been proved that administration of sevoflurane for the first two minutes of reperfusion effectively protects the heart against reperfusion injury in rats in vivo. Our aim was to investigate the duration of effective sevoflurane administration and its underlying mechanism in isolated rat hearts exposed to global ischemia/reperfusion (I/R) injury. Adult male Sprague-Dawley rats were randomly divided into six groups (n=12): a sham-operation group, an I/R group, and four sevoflurane postconditioning groups (S2, S5, S10, and S15). In the S2, S5, S10, and S15 groups, the duration times of sevoflurane administration were 2, 5, 10, and 15 min after the onset of reperfusion, respectively. The isolated rat hearts were mounted on the Langendorff system, and after a period of equilibrium were subjected to 40 min global ischemia and 120 min reperfusion. Left ventricular (LV) hemodynamic parameters were monitored throughout each experiment and the data at 30 min of equilibrium and 30, 60, 90, and 120 min of reperfusion were analyzed. Myocardial infarct size at the end of reperfusion (n=7 in each group) and the expression of myocardial phosphorylated Akt (p-Akt) after 15-min reperfusion were determined in a duplicate set of six groups of rat hearts (n=5 in each group). Compared with the I/R group, the S5, S10, and S15 groups had significantly improved left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), and the maximal rate of rise or fall of the LV pressure ($\pm dP/dt_{max}$), and decreased myocardial infarct size (P<0.05), but not the S2 group. After 15 min of reperfusion, the expression of p-Akt was markedly up-regulated in the S5, S10, and S15 groups compared with that in the I/R group (P<0.05), but not in the S2 group. Sevoflurane postconditioning for 5 min was sufficient to activate Akt and exert maximal cardioprotection against I/R injury in isolated rat hearts.

Key words: Sevoflurane postconditioning, Ischemia/reperfusion (I/R) injury, Cardioprotection, Duration of administration, Akt

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1 Introduction

Administering inhaled anesthetics, such as isoflurane and sevoflurane, at the onset of reperfusion, so called anesthetic postconditioning (APO), has been well demonstrated to provide cardioprotection against ischemia/reperfusion (I/R) injury in extensive animal experiments (Chen *et al.*, 2008; Redel *et al.*, 2009;

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Pravdic *et al.*, 2010). Because ischemia is usually unpredictable and happens suddenly, APO, which can be applied after ischemia, is attracting considerable clinical attention. However, the duration of effective APO administration both in patients and animals remains controversial, and the effect of APO against the myocardial I/R injury in patients is not as potent as in animals, partly due to the distinct administration time of APO adopted in different studies (Smul *et al.*, 2009).

Sevoflurane is widely used in cardiac surgery, since induction and recovery with sevoflurane are faster and smoother than those with other inhaled anesthetics (Wallin *et al.*, 1975; Sakai *et al.*, 2005). A meta-analysis showed that sevoflurane reduces the rate of myocardial infarct size and mortality in patients undergoing cardiac surgery, though the underlying mechanism remains unclear (Landoni *et al.*, 2007). Several recent studies have confirmed that sevoflurane postconditioning spares myocardial infarct size and improves contractile functions in I/R animals (Inamura *et al.*, 2010; Yao *et al.*, 2010b; Yu *et al.*, 2010; Zheng *et al.*, 2011). It is widely accepted that activating the phosphatidylinositol-3-kinase (PI3K)/Akt pathway is pivotal to cardioprotection by sevoflurane postconditioning against I/R injury (Yao *et al.*, 2010a; Yu *et al.*, 2010). However, there was no agreement among the different studies about the optimum duration of effective sevoflurane administration, even in similar myocardial I/R models. The protection of rat hearts by sevoflurane postconditioning was achieved within the first few minutes of reperfusion (Obal *et al.*, 2003; Inamura *et al.*, 2010). Longer administration (more than two minutes) of sevoflurane had no extra cardioprotective effects (Obal *et al.*, 2003), although it was reported to work after 15 min in some cases (He *et al.*, 2008; Yao *et al.*, 2010b). It seems that sevoflurane postconditioning needs time to activate downstream effectors, even though the exact duration of postconditioning and, especially, the underlying mechanisms remain unclear. Therefore, the purpose of this study was to investigate the exact duration of effective sevoflurane postconditioning in isolated rat hearts subjected to global I/R injury and to determine whether this time effect is related to the activation of Akt.

2 Materials and methods

2.1 Animals

Male Sprague-Dawley rats (230–250 g) were obtained from the Experimental Animal Center of Zhejiang Academy of Medical Sciences, China. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication Nos. 85–23, revised in 1996). The experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang University, China.

2.2 Reagents

Sevoflurane was purchased from the Maruishi Pharmaceutical Company (Osaka, Japan) and 2,3,5-triphenyltetrazolium chloride (TTC) from the Sigma-Aldrich Inc. (USA). Rabbit monoclonal Akt and phospho-Akt (p-Akt, Ser^{473}) antibodies were purchased from the Cell Signaling Technology (USA). Unless indicated otherwise, all other chemicals were of analytical purity.

2.3 Isolated perfused rat heart preparation

Rats of 230–250 g were anesthetized [40 g/L chloral hydrate, 8 ml/kg intraperitoneal (i.p.)] and heparinized (500 U/kg, i.p.). After median sternotomy, the heart was rapidly isolated and perfused on the Langendorff apparatus at (37±0.1) °C with a constant pressure (80 mmHg) using Krebs-Henseleit (K-H) buffer composed of (mmol/L): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.25, and glucose 10, and equilibrated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). A latex, fluid-filled balloon was introduced into the left ventricle via the left atrium, and the balloon catheter was linked to a pressure transducer connected to the physiological signal acquisition system (PowerLab, ADInstruments Shanghai Trading Co., Ltd., China) to monitor the contractile function. At the beginning of perfusion, the left ventricular end-diastolic pressure (LVEDP) was adjusted to 4–6 mmHg. All hearts were allowed to equilibrate for 30 min. After that, the flow in the I/R group was turned off for 40 min to elicit global ischemia, and then the hearts were reperfused for 120 min. Left ventricular developed pressure (LVDP),

LVEDP, the maximal rate of rise or fall of the LV pressure ($\pm dP/dt_{max}$), and the heart rate (HR) were monitored throughout the experimental period. Coronary flow (CF) was measured after reperfusion for 30, 60, 90, and 120 min by collecting the coronary effluent.

2.4 Experimental protocol

After equilibration, hearts were randomly assigned to the following six groups (n=7 in each group): a sham-operated group, continuously perfused with K-H buffer for 160 min; an I/R group (I/R), subjected to global ischemia for 40 min followed by 120 min reperfusion; and sevoflurane postconditioning groups treated for 2, 5, 10, or 15 min (S2, S5, S10, or S15, respectively), subjected to I/R and receiving 2.5% sevoflurane [1.0 minimum alveolar concentration (MAC) at 37 °C in rats]. The concentration in K-H buffer was determined using a gas chromatograph (Perkin-Elmer, Norwalk, CT) before the sevoflurane entered the aorta for 2, 5, 10, or 15 min at the onset of reperfusion. Sevoflurane was delivered into the K-H buffer via a Sevotec 3 vaporizer (Datex-Ohmeda, Tewksbury, MA) and equilibrated with an air bubbler for 15 min before opening the three-way stopcock for reperfusion.

2.5 Determination of myocardial infarct size

At the end of reperfusion, hearts were frozen at −20 °C for 2 h and then cut transversely into 5 slices. The slices were stained at 37 °C for 10 min in 1% TTC in 0.1 mol/L phosphate buffer and then incubated in 10% formalin to identify the viable (red) and infarct (pale) tissue. The infarct size was determined by planimetry with ImageJ 1.37 from NIH and is expressed as a percentage of the whole LV slices (all five slices were taken into account by summing the values).

2.6 Western blot analysis

Another six groups of rat hearts (n=5 in each group) were perfused using the experimental protocol described above. LV tissue samples were taken at 15 min reperfusion and immediately frozen in liquid nitrogen. The tissue samples were homogenized in ice-cold radio immunoprecipitation assay (RIPA) buffer and the protein concentration was determined using bicinchoninic acid (BCA) assay kits (Beyotime Institute of Biotechnology, China). Proteins (50 μg) were loaded onto gels and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked and subsequently incubated for 24 h at 4 °C with anti-Akt and p-Akt. It was then incubated with fluorescence labeled mouse anti-rabbit IgG. Immune complex was detected using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The quantitative protein band densities were assayed by ImageJ 1.37 and expressed as the ratio of GAPDH band density.

2.7 Statistical analysis

All values are expressed as mean±standard deviation (SD). The statistical analysis was performed using SPSS 13.0 (Chicago, USA). The variables of LV contractile function were analyzed by two-way analysis of variance (ANOVA). All other data were analyzed by one-way ANOVA followed by post-hoc Tukey tests. The differences were considered significant when P<0.05.

3 Results

3.1 Effect of different durations of sevoflurane administration on the left ventricular (LV) contractile function in rat hearts exposed to ischemia/reperfusion (I/R)

After 30 min equilibration, there were no significant differences among groups for any of the parameters of the LV contractile function (P>0.05). At 30 min reperfusion, LVDP, $\pm dP/dt_{max}$, and CF were markedly decreased and LVEDP was increased in the I/R group compared with the corresponding values in the sham group (P<0.01). All of these effects were ameliorated in the S5, S10, and S15 groups (P<0.05 vs. I/R group), but not in the S2 group. These improvements in contractile functions and CF in the S5, S10, and S15 groups were maintained during the whole reperfusion period. There were no significant differences among the S5, S10, and S15 groups for any of the LV contractile function parameters throughout the experiment. There were no significant differences in HR among groups throughout the experiment (P>0.05) (Table 1).

Table 1 Effect of sevoflurane postconditioning for 2, 5, 10, or 15 min (S2, S5, S10, or S15, respectively) on the left ventricular (LV) contractile functions of rat hearts exposed to ischemia/reperfusion (I/R)

Group	LVEDP (mmHg)	LVDP (mmHg)	$+dP/dt_{max}$ (mmHg/s)	$-dP/dt_{max}$ (mmHg/s)	HR (beat/min)	CF (ml/min)
Baseline						
Sham	6.3±0.3	104±3.0	3390±263	2424±257	293±13	9.0±0.1
I/R	7.0±0.1	98±11.0	2816±259	2256±247	282±26	8.9±0.3
S2	6.3±2.0	101±19.0	3168±322	2149±278	294±26	9.0±0.9
S5	5.6±0.8	112±14.0	3017±385	2132±203	290±9	8.8±0.4
S10	6.0±0.8	111±11.0	3227±236	2173±200	280±17	9.0±0.1
S15	5.6±1.0	110±10.0	3133±228	2263±287	291±12	9.3±0.7
Reperfusion 30 min						
Sham	6.8±2.7	70±15.0	2623±211	1745±181	278±27	6.8±0.9
I/R	46.0±7.0**	23±5.7**	891±146**	561±96**	249±10	3.0±0.5**
S2	47.0±7.0**	24±10.0**	1015±47**	658±73**	259±26	3.2±1.0**
S5	22.0±6.5$^{**\#\#}$	52±7.0$^{\#\#}$	1316±78$^{**\#}$	1013±127$^{**\#}$	266±18	5.3±0.4$^{\#\#*}$
S10	25.0±6.0$^{**\#}$	54±7.0$^{\#\#}$	1419±167$^{**\#}$	988±81$^{*\#\#}$	254±27	5.5±0.8$^{\#\#}$
S15	26.5±13.0$^{**\#\#}$	49±16.0$^{\#}$	1300±93$^{**\#}$	920±66$^{*\#\#}$	288±21	5.5±1.4$^{\#}$
Reperfusion 60 min						
Sham	7.8±3.0	63.0±9.5	2365±277	1587±148	275±13	6.3±1.0
I/R	40.0±3.6**	30.0±5.0**	929±181**	632±66**	238±9	3.5±0.8**
S2	36.0±7.0**	36.7±8.2**	927±132**	686±59**	242±17	4.4±1.0**
S5	18.0±4.0$^{\#\#}$	56.0±10.0$^{\#\#}$	1444±289$^{**\#}$	1049±170$^{**\#\#}$	245±16	5.4±1.4$^{\#\#}$
S10	23.0±4.0$^{*\#\#}$	54.0±4.0$^{\#\#}$	1538±210$^{**\#}$	947±115$^{**\#\#}$	265±14	5.3±5.0$^{\#\#}$
S15	20.0±9.0$^{\#\#}$	51.0±8.0$^{\#\#}$	1437±249$^{**\#}$	930±46$^{**\#\#}$	267±15	5.3±0.7$^{\#}$
Reperfusion 90 min						
Sham	7.8±3.0	53.5±8.5	2179±128	1472±222	276±9	5.5±0.7
I/R	36.5±6.0**	28.0±7.0**	804±143*	532±40*	232±5	3.4±0.8**
S2	32.0±7.0**	36.6±8.8	930±96*	654±105*	233±22	4.5±1.0
S5	16.0±4.0$^{**\#\#}$	48.4±12.5$^{\#}$	1380±149$^{*\#}$	919±88$^{\#\#}$	243±16	5.1±0.7$^{\#\#}$
S10	21.0±5.0$^{**\#}$	50.0±4.0$^{\#\#}$	1486±134$^{*\#}$	900±85$^{\#\#}$	252±18	5.0±0.4$^{\#\#}$
S15	18.0±7.0$^{**\#\#}$	47.0±4.0$^{\#\#}$	1415±233$^{*\#}$	870±43$^{\#\#}$	256±22	4.8±0.4$^{\#\#}$
Reperfusion 120 min						
Sham	8.6±3.0	47.6±7.7	2036±65	1395±121	269±7	4.8±0.6
I/R	37.0±5.0**	24.0±5.0**	700±73*	440±66*	232±11	2.9±0.5**
S2	32.0±10.0**	32.5±10.0	809±151*	657±128*	230±21	3.7±0.7
S5	15.7±3.6$^{\#\#}$	44.0±10.0$^{\#}$	1233±170$^{*\#}$	915±68$^{*\#}$	247±17	4.4±0.9$^{\#\#}$
S10	20.0±5.0$^{**\#\#}$	45.0±6.0$^{\#\#}$	1320±127$^{*\#}$	819±86$^{*\#}$	244±20	4.8±0.3$^{\#\#}$
S15	17.0±7.0$^{**\#\#}$	43.0±2.0$^{\#\#}$	1517±237$^{*\#}$	816±60$^{*\#}$	260±22	4.1±0.4$^{\#\#}$

Data are expressed as mean±SD, n=7. LVDP: left ventricular developed pressure; LVEDP: left ventricular end-diastolic pressure; $\pm dP/dt_{max}$: the maximal rise or fall rate of left ventricular pressure; CF: coronary flow; HR: heart rate. * P<0.05, ** P<0.01 vs. the sham group; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01 vs. the I/R group

3.2 Effect of different durations of sevoflurane administration on the infarct size in rat hearts exposed to I/R

After 120 min reperfusion, myocardial infarct size was significantly increased in the I/R group compared with that in the sham group [(36.4±3.9)% vs. (1.5±0.5)%, P<0.01] (Fig. 1), but was markedly decreased in the S5, S10, and S15 groups [(19±6.5)%, (18±5.6)%, and (22±3.6)%, vs. (36.4±3.9)%, P<0.01]. There were no significant differences in infarct size among the S5, S10, and S15 groups. However, the infarct size in the S2 group (33.9±6.9)% was not decreased compared with that in the I/R group (P>0.05).

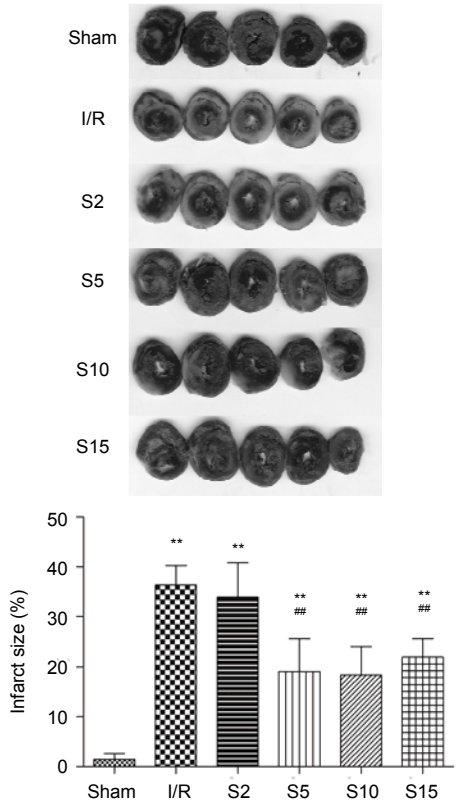

Fig. 1 Effect of sevoflurane postconditioning for 2, 5, 10, or 15 min (S2, S5, S10, or S15, respectively) on infarct size in rat hearts exposed to I/R

Data are expressed as mean±SD (n=7). $^{**}P$<0.01 vs. the sham group; $^{\#\#}P$<0.01 vs. the I/R group

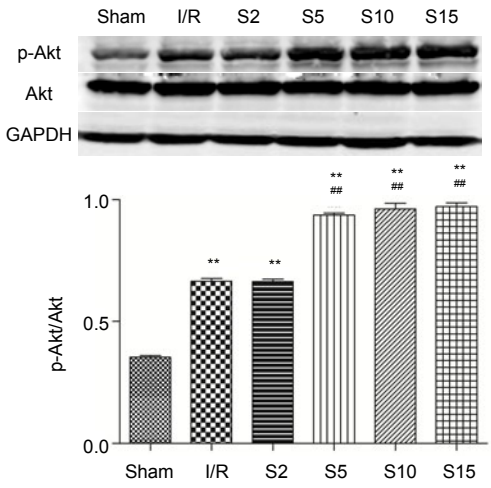

Fig. 2 Effect of sevoflurane postconditioning for 2, 5, 10, or 15 min (S2, S5, S10, or S15, respectively) on the p-Akt expression in rat hearts exposed to I/R

Data are expressed as mean±SD (n=6). $^{**}P$<0.01 vs. the sham group; $^{\#\#}P$<0.01 vs. the I/R group

3.3 Effect of different durations of sevoflurane administration on the expression of p-Akt in rat hearts exposed to I/R

After 120 min reperfusion, the expression of myocardial p-Akt was significantly increased in the I/R group compared with that in the sham group (P<0.01), and was further increased in the S5, S10, and S15 groups (P<0.01 vs. the I/R group) (Fig. 2). Myocardial p-Akt expression did not differ significantly among the S5, S10, and S15 groups. However, expression in the S2 group was not increased compared with that in I/R group (P>0.05).

4 Discussion

4.1 Cardioprotection effect of sevoflurane postconditioning

Restoration of the blood flow to the ischemic heart as early as possible is the only way to salvage patients exposed to myocardial ischemia. However, reperfusion itself has the potential to produce additional injury to the ischemic heart, called myocardial I/R injury. Sevoflurane postconditioning has been reported to reduce myocardial infarct size and ameliorate cardiac functions in I/R rat hearts (Siegmund *et al.*, 1997; Chen *et al.*, 2008; Inamura *et al.*, 2010). Some studies agreed that 3% sevoflurane postconditioning for 15 min conferred significant cardioprotection in isolated I/R rat hearts (He *et al.*, 2008; Yao *et al.*, 2010b), but our recent study found that 2.5% sevoflurane (1 MAC) postconditioning for 10 min was sufficient to improve myocardial function and reduce infarct size after global I/R in isolated rat hearts (Yu *et al.*, 2010).

4.2 Appropriate concentration of sevoflurane postconditioning

One MAC of sevoflurane has been shown to significantly protect against myocardial I/R injury, whereas a lower postconditioning concentration (0.75 MAC) gave no cardioprotection (Obal *et al.*, 2001). Interestingly, the cardioprotection was not further enhanced when the administering concentration was over 1 MAC. Therefore, the appropriate concentration of sevoflurane postconditioning in rat hearts seems to be 2.5% (1 MAC).

4.3 Duration of effective sevoflurane administration

The threshold of the duration of effective sevoflurane administration remains controversial. In this work, we found that 2.5% sevoflurane postconditioning for 5 min significantly improved the LV functions and reduced myocardial infarct size after global I/R in isolated rat hearts. This cardioprotection was not observed after sevoflurane postconditioning for 2 min, and was not enhanced as the duration of administration was extended to 10 or 15 min. These results indicate that the first 5 min of reperfusion is the vital time window for cardioprotection induced by sevoflurane postconditioning. This timing is fortunate because myocardial I/R injury is usually triggered at the onset of reperfusion, especially during the first 2–5 min of reoxygenation (Siegmund *et al.*, 1997; Ladilov *et al.*, 1998). We found that a short duration of sevoflurane administration, such as two minutes, might not be enough to reduce I/R injury in cardiomyocytes. This result is in contrast to the results of previous in vivo experiments in rats (Obal *et al.*, 2003). However, we used the global I/R model and a longer I/R period (40 min/120 min), whereas Obal *et al*. (2003) used the regional I/R model and a shorter I/R period (25 min/90 min). Therefore, we suppose that the cardiac injury in our I/R model was probably more drastic and that a greater duration of sevoflurane administration was needed to produce the cardioprotective effect. Moreover, the blood-gas partition coefficient of sevoflurane is only 0.59 (Kazama and Ikeda, 1988), which means that a saturated concentration of sevoflurane might be reached and produce maximal cardioprotection in only 5 min. Further effective sevoflurane may not accumulate once the blood has become saturated. Although we cannot determine the minimal duration of effective sevoflurane postconditioning in the current study because we did not test for 3 or 4 min, it appears that cardioprotection against reperfusion injury was maximal within only 5 min of sevoflurane postconditioning in our current study using isolated rat hearts.

4.4 Sevoflurane postconditioning needs time (5 min or more) to activate Akt

Activation of the PI3K/Akt pathway is well-known to mediate the cardioprotection of APO (Chiari *et al.*, 2005; Feng *et al.*, 2006; Yu *et al.*, 2010). When I/R rat hearts are postconditioned with sevoflurane, Akt is phosphorylated and activated by PI3K, and then blocks the expression of several pro-apoptotic proteins, such as p53, Bad, Bax, and caspases, to promote cell survival (Cantley, 2002; Yu *et al.*, 2010). In our current study, the expression of myocardial p-Akt was up-regulated by sevoflurane administration for 5, 10, and 15 min to the same level, but not by 2 min of postconditioning. This result suggests that sevoflurane postconditioning needs time to activate Akt and exerts cardioprotection against I/R injury in isolated rat hearts. Moreover, there is a certain upper limit in the phosphorylation of Akt induced by sevoflurane postconditioning, and the protection, once triggered, is not enhanced further by extending the duration of sevoflurane administration.

5 Conclusions

In summary, these results suggest that in isolated rat hearts, sevoflurane postconditioning for 5 min is sufficient to activate myocardial Akt against I/R injury and that this cardioprotection is not changed extension of the duration of sevoflurane administration.

Compliance with ethics guidelines

Yuan-yuan YAO, Man-hua ZHU, Feng-jiang ZHANG, Chuan-yun WEN, Lei-lei MA, Wen-na WANG, Can-can WANG, Xian-bao LIU, Li-na YU, Ling-bo QIAN, Jian-an WANG, and Min YAN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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