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**Acute Memory Phase of Sevoflurane Preconditioning is Associated  
with Sustained Translocation of PKC- $\alpha$  and  $\epsilon$ , but not  $\delta$ , in  
Isolated Guinea Pig Hearts**

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Short Title: Memory phase of sevoflurane preconditioning

## Summary

*Background and objective:* Anaesthetic preconditioning exerts cardioprotective effects by reducing infarct size and improving recovery of contractile function after ischemia-reperfusion. The interval between brief exposure to volatile anaesthetic and sustained ischemia, the acute memory phase, is dependent on intracellular signaling mediating this cardioprotection. Intramyocyte translocation of protein kinase C is known to be a key mediator in anaesthetic preconditioning. We examined the relationship between the time frame of the acute memory phase of sevoflurane preconditioning and intramyocyte translocation of protein kinase C- $\alpha$ ,  $\delta$  and  $\epsilon$  to the particulate fraction.

*Methods:* Isolated perfused guinea pig hearts were subjected to 30 min ischemia and 120 min reperfusion. Anaesthetic preconditioning was elicited with one minimum alveolar concentration sevoflurane for 10 min. Washout times of 10, 30, 60, and 90 min were studied. Contractile recovery was assessed monitoring left ventricular developed pressures. Infarct size was determined by triphenyltetrazolium chloride staining. Translocation of protein kinase C was examined by Western blot analysis.

*Results:* After ischemia-reperfusion, left ventricular developed pressure recovered to a greater degree with anaesthetic preconditioning compared with control for washout times of 10 and 30 min, but not 60 and 90 min. Similarly, infarct size was reduced for washout times of 10 and 30 min, but not 60 and 90 min. Sustained translocation of protein kinase C- $\alpha$  and  $\epsilon$ , but not  $\delta$ , was associated with the time frame of the acute memory phase.

*Conclusions:* The acute memory phase of sevoflurane preconditioning is limited to less than 60 min. Sustained translocation of protein kinase C- $\alpha$  and  $\epsilon$ , but not  $\delta$ , correlate with this acute memory phase of sevoflurane preconditioning.

**Key Words:** sevoflurane, myocardial preconditioning, protein kinase C

## Introduction

Anaesthetic preconditioning (APC) exerts cardioprotective effects by reducing infarct size and improving recovery of contractile function after ischemia-reperfusion [1-4]. The interval between brief exposure to volatile anaesthetic and sustained ischemia, the acute memory phase, is dependent on intracellular signaling mediating the APC cardioprotection. Beyond the acute memory phase, the beneficial effects of volatile anaesthetic against ischemia-reperfusion injury are lost. Kersten et al. [5] demonstrated that isoflurane produced an acute memory phase with a duration of at least 30 min in an *in vivo* dog study. In the same experimental setting, Toller et al. [6] reported that the acute memory phase of one minimum alveolar concentration (1 MAC) sevoflurane for 30 min disappeared by 30 min after discontinuation of sevoflurane. The time frame of the acute memory phase for sevoflurane APC in other animal models is not known.

Protein kinase C (PKC) activation has been shown to be a pivotal signaling step in APC [7-9]. Sevoflurane APC is mediated by PKC- $\epsilon$ , but not  $\delta$ , in isolated guinea pig hearts [10]. In contrast, translocation of PKC- $\delta$ , but not  $\epsilon$ , to mitochondria has been shown to play a role in isoflurane APC [11]. Immunocytochemical analysis demonstrated that propofol translocates PKC- $\epsilon$  to intercalated discs and the sarcolemma, and PKC- $\delta$  to the perinuclear region in rat ventricular myocytes [12]. Although PKC appears to play a role in APC, the relationship between isoform-specific PKC translocation and the duration of the acute memory phase is not known. The purpose of this study was to identify the time frame of the acute memory phase of sevoflurane APC in isolated guinea pig hearts. Further, the relationship between PKC isoform ( $\alpha$ ,  $\delta$  and  $\epsilon$ ) translocation and the duration of the sevoflurane APC acute memory phase was studied. As volatile anaesthetics can be

administrated with relatively low toxicity, elucidating the signaling mechanisms underlying APC may prove clinically useful during cardiac and non-cardiac surgeries [13-15].

## Methods

This study was conducted in accordance with the Guidelines for Animals Research at Osaka Dental University (ODU), and with the approval of the Animal Experiment Committee of ODU, Osaka, Japan. Male Hartley guinea pigs were fed Lab Diet (RC4<sup>TM</sup>, Oriental Yeast, Tokyo, Japan) and given water *ad libitum*.

### *Isolated Heart Perfusion and Measurement of Function*

Animals weighing 650-700g were given heparin (1000 units i.p.), then anaesthetized with pentobarbital (60 mg kg<sup>-1</sup> i.p.). Hearts were excised and immediately arrested in cold isosmotic saline containing 20 mmol L<sup>-1</sup> KCl. Aortas were cannulated and the isolated hearts were retrogradely perfused at 70 mmHg on a nonrecirculating isovolumic perfused heart apparatus, using a Krebs-Henseleit (KH) perfusate (mmol L<sup>-1</sup>): 118 NaCl, 4.0 KCl, 2.52 CaCl<sub>2</sub>, 24.8 NaHCO<sub>3</sub>, 1.7 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 11.0 glucose, 0.5 EDTA and 8 units L<sup>-1</sup> insulin. The perfusate was insufflated continuously with O<sub>2</sub> 95% and CO<sub>2</sub> 5%. Hearts were paced at 240 beats min<sup>-1</sup> using platinum-tipped electrodes connected to a Grass Instruments (Quincy, MA, USA) SD-5 stimulus generator. Left ventricular developed pressure (LVDP) was measured from a 2.5 French, mikro-tip<sup>®</sup> catheter transducer (SPR-524; Millar Instruments, Inc., Houston, TX, USA) passed into a compliant latex balloon inserted into the LV, and recorded on a PowerLab 2/20 Data Recording System (ADInstruments, Hayward, Australia). The balloon was connected to a Y-adapter with one end used to advance the micromanometer and the other used to fill the LV balloon with bubble-free water to an end-diastolic pressure (LVEDP) of 10 mmHg. The maximum rate of increase of LV pressure (+dP/dt<sub>max</sub>) and the minimum rate of decrease of LV pressure (-dP/dt<sub>min</sub>) were calculated using Chart<sup>TM</sup>5 (ADInstruments). Coronary flow (CF) was measured by collecting effluent. Global ischemia was achieved by clamping the aortic inflow line. Heart temperature was

continuously monitored by a digital thermometer (PTW-100A, Unique Medical, Japan). During ischemia, hearts were maintained at 37 °C by enclosure in a water-jacketed air chamber. Warmed perfusate kept in the lower part of the chamber saturated the air with humidity and prevented cooling by evaporation.

### *Experimental Protocol*

Animals were assigned 7 groups (n=8 each; Figure 1). All hearts were subjected to 30 min and 120 min of reperfusion. After a 30 min equilibration, baseline LVDP, LVEDP and CF were recorded. Sevoflurane (1MAC; 2%) was insufflated by passing O<sub>2</sub> 95% and CO<sub>2</sub> 5% gas mixture for 10 min through a calibrated vaporizer (ACOMA, Tokyo, Japan). Coronary perfusate samples were collected anaerobically from the aortic cannula to measure sevoflurane concentration with an organic vapor sensor (OSP, Saitama, Japan). To define the limits of the acute memory phase of 1 MAC sevoflurane APC, washout times of 10 min (10 MIN), 30 min (30 MIN), 60 min (60 MIN) and 90 min (90 MIN) were studied. To examine the role of PKC during the acute memory phase, the PKC inhibitor chelerythrine (10µM, Alexis-Calbiochemicals, Tokyo, Japan), was administered for 20 min, starting 10 min before sevoflurane exposure in 30 MIN (30 MIN+CHE). Chelerythrine was dissolved in distilled water, and added to the KH perfusate to a final concentration of 10 µM.

### *Determination of Myocardial Infarct Size*

Infarct size was determined as previously described [8]. Briefly, at the end of experiments, the hearts were quickly frozen and sliced into 2 mm thick transverse sections. Each slice was incubated with 1% triphenyltetrazolium chloride (Sigma Chemicals) and was digitally photographed and the necrotic area was determined using Adobe Photoshop® CS

(Adobe, CA, USA), then expressed as a fraction of left ventricle.

### *Western Blot Analysis*

Translocation of PKC isoforms in response to preconditioning was assessed by Western Blot analysis. Myocardial tissue samples were collected before ischemia (n=4 in each group), and homogenized in TEE buffer (50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3%  $\beta$ -mercaptoethanol, and 50  $\mu\text{g ml}^{-1}$  PMSF, pH 7.5). Homogenate was centrifuged at 1,000g and 4 °C for 5 min. Supernatants were re-centrifuged at 10,000g and 4 °C for 15 min. Homogenates were centrifuged at 100,000g for 60 min and the supernatant was frozen at -80°C (cytosolic fraction). Pellets were resuspended in TEET buffer (TEE buffer with 0.2% Triton X-100), incubated for 60 min and centrifuged at 100,000g for 60min (particulate fraction). Protein concentrations were estimated with a Bradford assay. Equivalent amounts (50  $\mu\text{g}$ ) of protein samples were loaded and separated on a 7.5 % SDS-PAGE gel, then electrically transferred to a PVDF membrane (Millipore Co., Billerica, USA). After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), membranes were incubated for 2 hr at 4 °C in TBS-T containing 5% milk and 1:1000 dilution of rabbit polyclonal antibodies for PKC- $\alpha$  and PKC- $\epsilon$ , and PKC- $\delta$  (C-20, C-15, and C-20, Santa Cruz Biotechnology, USA). Membranes were incubated with a 1:1000 dilution of horseradish peroxidase-labeled anti-rabbit immunoglobulin G (NA 934V, GE Healthcare, UK). Bound antibody signals were detected with enhanced chemiluminescence (Pierce Biotechnology, IL, USA) and visualized using VersaDoc 5000<sup>®</sup> Imaging System (Bio-Rad). Quantitative analysis of the band densities was performed by Quantity One<sup>®</sup> software (Bio-Rad).

### *Statistics*

All data are expressed as mean $\pm$ SD. Two-factor repeated-measures analysis of

variance was used to evaluate differences over time between groups. If differences were observed, a Tukey post-hoc test was used to confirm the significance of differences between groups. The differences in expressions of PKC cytosolic and particulate fractions were determined by unpaired Student's *t* test. A value of  $p < 0.05$  was considered statistically significant.

## Results

The concentration of sevoflurane in the coronary perfusate after 10 min of exposure was  $0.41 \pm 0.08$  mM. Sevoflurane was not detected in the effluent during the baseline, ischemic, and reperfusion periods.

### *Hemodynamics*

Baseline and pre-ischemia LVDP, LVEDP,  $+dP/dt_{max}$ ,  $-dP/dt_{min}$  and CF were similar among the 7 groups (Table 1). Recovery of LVDP and LVEDP was significantly greater throughout reperfusion in 10 MIN and 30 MIN, but not 60 MIN and 90 MIN, when compared with **control (CTL)**. Recovery of LVDP and LVEDP in 30 MIN was abolished by administration of CHE. **Although CF significantly decreased after ischemia-reperfusion compared to baseline in all groups, the difference was only statistically significant in 60 MIN, 90 MIN and 30 MIN+CHE ( $p < 0.05$ ). Nevertheless, there was no significant difference in CF among all groups throughout the experiment at each time point. This suggests that changes in CF do not account for the improved contractile recovery of 10 MIN and 30 MIN.**

Recovery of  $+dP/dt_{max}$  and  $-dP/dt_{min}$  was significantly greater throughout reperfusion in 10 MIN and 30 MIN, but not in 60 MIN and 90 MIN, when compared with CTL (Table 1).

### *Myocardial Infarct Size*

**After ischemia-reperfusion, infarct size was significantly reduced by APC compared with CTL for washout times of 10 and 30 min (10 MIN:  $21 \pm 7\%$ , 30 MIN:  $25 \pm 10\%$  vs. CTL:  $42 \pm 5\%$ ,  $p < 0.05$ ), but not 60 and 90 min (60 MIN:  $37 \pm 9\%$ , 90 MIN:  $41 \pm 12\%$ ,  $p = NS$  vs. CTL). This cardioprotective effect was abolished by CHE (30 MIN+CHE:  $46 \pm 13\%$ ,  $p = NS$  vs. CTL). CHE treatment alone did not affect infarct size (CTL+CHE:  $47 \pm 10\%$ ) (Figure 2).**

*Western Blot analysis*

The densities of cytosolic and particulate fractions of PKC- $\alpha$ ,  $\epsilon$  and  $\delta$  were normalized against the corresponding densities of CTL (Figure 3). After sevoflurane exposure, PKC- $\alpha$  and  $\epsilon$ , but not  $\delta$  were translocated to the particulate fraction until 30 min, but not 60 and 90 min. Chelerythrine abolished this translocation of PKC- $\alpha$  and  $\epsilon$  in 30MIN+CHE.

## Discussion

The present study defined for the first time the duration of the acute memory phase of sevoflurane APC and its relationship to intramyocyte translocation of PKC isoforms in the isolated guinea pig heart model. Sevoflurane APC protects against ischemia-reperfusion injury, but this cardioprotective effect is restricted to an acute memory phase of less than 60 min. Sustained translocation of PKC- $\alpha$  and  $\epsilon$ , but not  $\delta$ , to the particulate fraction is associated with this protective time frame. The PKC inhibitor, chelerythrine, inhibited both the sevoflurane-induced reduction in infarct size and translocation of PKC- $\alpha$  and  $\epsilon$ . This suggests that translocation of PKC- $\alpha$  and  $\epsilon$  mediates the acute memory phase of sevoflurane APC.

In acute ischemic preconditioning, the infarct size limiting effect is lost if the memory phase is prolonged beyond 1 hr in pigs [16], and 1-2 hr in rabbits [17] or dogs [18]. In APC, Toller et al. reported that the acute memory phase of 1 MAC sevoflurane for 30 min disappeared by 30 min after discontinuation in an *in vivo* dog model [6]. In contrast, 1 MAC isoflurane has been shown to exert APC after the 30-min washout period in the same dog model [5]. The present study demonstrates that the acute memory phase associated with sevoflurane APC is present for greater than 30 min in guinea pig hearts.

Translocation of PKC isoforms has been implicated in APC, as well as ischemic and ethanol-induced preconditioning [7,8,19-21]. Upon activation, PKC isoforms translocate to different subcellular components in the myocyte where they bind to anchoring molecules termed receptors for activated C kinase (RACKs) [22]. Isoform-selective RACKs are located on a variety of subcellular structures, including membranes and cytoskeletal elements. After binding to their selective RACKs, activated PKC isoforms phosphorylate protein substrates which propagate the preconditioning cardioprotective signaling cascade. Using isoform-

specific inhibitors, Novalija et al. [10] demonstrated that sevoflurane APC preconditioning is mediated by PKC- $\epsilon$ , but not  $\delta$ , in isolated guinea pig hearts. However, it has been reported that PKC- $\delta$ , rather than PKC- $\epsilon$ , is involved in acute memory phase of isoflurane APC by modulating sarcolemmal  $K_{ATP}$  channels in rat cardiomyocytes [23]. In the present study, PKC- $\alpha$  and  $\epsilon$ , but not  $\delta$ , were found to play a role in the acute memory phase of sevoflurane APC in guinea pig hearts.

Ischemic preconditioning was not observed in either PKC- $\epsilon$  and PKC- $\delta$  knockout mice [24,25]. This suggests that both PKC isoforms are required for ischemic preconditioning. Miyamae et al. [20] found that regular ethanol consumption induced chronic preconditioning that was associated with sustained intramyocyte translocation of PKC- $\epsilon$ , but not PKC- $\alpha$  or  $\delta$ , in guinea pig. Inagaki et al. [26] demonstrated that the timing of activation of PKC- $\epsilon$  and PKC- $\delta$  affects cardioprotection by acute ethanol-induced preconditioning in mice. They reported that PKC- $\epsilon$  activation during ischemia is cardioprotective, whereas PKC- $\delta$  activation in the middle of reperfusion is detrimental. Further, they found that initial PKC- $\delta$  activation leads to subsequent PKC- $\epsilon$  activation through release of adenosine [26]. In an isolated rat heart model of isoflurane APC, PKC- $\delta$  translocation to mitochondria and phosphorylation during the acute memory phase may play a role in transferring APC signaling to mitochondrial  $K_{ATP}$  channels [11]. In the present study, APC-induced translocation of PKC- $\delta$  to the particulate fraction (which includes the mitochondrial fraction) before ischemia was not observed. This finding is in agreement with those of Novalija et al. They found that sevoflurane APC is mediated by PKC- $\epsilon$ , but not  $\delta$ , in isolated guinea pig hearts. Differences in the importance of PKC isoforms in mediating preconditioning and its acute memory phase may be due to differences in the preconditioning stimulus or species studied.

Recently, PKC- $\alpha$  has been found to play a role in sevoflurane APC through translocation to mitochondria in rat trabecula [27]. Using immunofluorescent staining, this study demonstrated that 3.8 % sevoflurane exposure for 15 min with 15 min washout translocated PKC- $\alpha$  to mitochondria. Further, PKC- $\alpha$  translocation was dependent on reactive oxygen species. The present study demonstrates that inhibition of PKC- $\alpha$  translocation by CHE during the acute memory phase abolished sevoflurane APC cardioprotection. This suggests that PKC- $\alpha$  may play a role in sevoflurane APC and its acute memory phase.

The following study limitations should be acknowledged. We did not directly measure PKC activity. The extent and duration of PKC redistribution determined by Western blot analysis may not truly reflect the amount of PKC activity which is more accurately determined after subcellular fractionation of the cell [28]. In addition, the methodology of our study does not allow us to make a definite link between the time frame of cardioprotection and translocation of PKC isoforms. The dose of CHE used in the present study has been previously found to abolish PKC activity [20]. CHE abolished both improvement of contractile dysfunction after ischemia-reperfusion and translocation of PKC- $\alpha$  and  $\epsilon$ , but it is unclear which isoform plays a more important role in this acute memory phase, as CHE is not isoform-specific.

In conclusion, the acute memory phase of sevoflurane APC is lost between 30 min and 60 min after sevoflurane exposure in an isolated guinea pig heart model. Sustained intramyocyte translocation of PKC- $\alpha$  and  $\epsilon$ , but not  $\delta$ , to the particulate fraction plays a role in mediating this sustained cardioprotection.

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**Table Legend**

Table 1: Data are presented as mean  $\pm$  SD. LVDP=left ventricular developed pressure; LVEDP=left ventricular end-diastolic pressure; CF=coronary flow;  $+dP/dt_{\max}$ =maximum rate of increase of LV pressure;  $-dP/dt_{\min}$ = maximum rate of increase of LV pressure; CTL=control; SEVO=sevoflurane; CHE=chelerythrine; \* $p<0.05$  vs. CTL, n=8 for each group

## Figure Legends

Figure 1: Schematic illustration of the experimental protocol of this study. All hearts were subjected to 30 min global ischemia followed by 120 min reperfusion. Anaesthetic preconditioning was elicited by administration of sevoflurane (2% or 1 MAC) for 10 min before ischemia. To determine the critical limits of efficacy of APC in this model, washout times of 10, 30, 60, and 90 min were studied (10 MIN, 30MIN, 60MIN, 90MIN). PKC inhibitor, chelerythrine (10 $\mu$ M) was administered for 20 min, starting 10 min before sevoflurane exposure in 30 MIN (30 MIN+CHE).

CTL=control; CHE=chelerythrine, a PKC inhibitor

Figure 2: Infarct size as a percentage of left ventricle in 7 groups. After ischemia-reperfusion, infarct size was significantly reduced by APC compared with control for washout times of 10 and 30 min, but not 60 and 90 min. This cardioprotective effect was abolished by CHE. CHE treatment alone did not affect infarct size. Closed circles: individual infarct size data points; open circles: mean infarct size; bars: mean  $\pm$  SD. Data are presented as mean  $\pm$  SD. \*p<0.05 vs. CTL

Figure 3: Western blot analysis of PKC- $\alpha$ ,  $\epsilon$ , and  $\delta$  from left ventricular samples before ischemia in control and sevoflurane-treated hearts (n=4 for each group). PKC- $\alpha$  and  $\epsilon$ , but not  $\delta$ , were translocated to the particulate fraction until 30 min, but not 60 and 90 min, after sevoflurane exposure. \*p<0.05 vs. 10MIN cytosolic fraction, #p<0.05 vs. 30MIN cytosolic fraction, C=cytosolic fraction; P=particulate fraction