Isoflurane-induced post-conditioning in senescent hearts is attenuated by failure to activate reperfusion injury salvage kinase pathway

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A Dissertation Submitted to The Graduate School of Ajou University in Partial Fulfillment of the Requirements for The Degree of Master of Medicine

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February, 2013

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November, 19th, 2012

– ABSTRACT –

Isoflurane-induced post-conditioning in senescent hearts is attenuated by failure to activate reperfusion injury salvage kinase pathway

Background: We investigated the cardioprotective effects of isoflurane administered at the onset of reperfusion in senescent rat in vivo, and the activation of the reperfusion injury salvage kinase (RISK) pathway to address a possible mechanism underlying age-related differences.

Methods and results: Male Wistar rats were assigned to age groups (young, 3–5 months; old, 20–24 months), and randomly selected to receive isoflurane (1 minimum alveolar concentration) or not for 3 min before and 2 min after reperfusion (ISO postC). Rats were subjected to coronary occlusion for 30 min followed by 2 h of reperfusion. Western blot analysis was used to assess the phosphorylation of extracellular signal-regulated kinase (ERK1/2), Akt, and GSK3β 15 min after reperfusion. Brief administration of isoflurane 3 min before and 2 min after the initiation of early reperfusion reduced infarct size (56 ± 8% of left ventricular area at risk, mean ± standard deviation) compared with controls (68 ± 4%) in young rats, but had no effect in old rats (56 ± 8% in ISO postC and 56 ± 10% in control, respectively). Phosphorylation of ERK1/2, Akt, and GSK3β were increased in the young ISO postC group but not in the old ISO postC group compared with control groups of the respective ages.

Conclusions: We demonstrated that isoflurane postconditions the heart in young but not in senescent rats. Failure to activate RISK pathway may contribute to attenuation of isoflurane-induced post-conditioning effect in senescent rats.

Keywords: Heart, isoflurane, post-conditioning, RISK pathway

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Ⅰ. INTRODUCTION

Ischemic pre-conditioning, or a brief exposure to ischemia prior to sustained ischemia, has been considered one of the strongest forms of endogenous cardioprotection ever since the first demonstration of ischemic pre-conditioning in the dog myocardium.(Murry et al., 1986) However, pre-conditioning is less clinically relevant because of the unpredictability of the timing of myocardial infarction. Zhao et al. demonstrated that brief episodes of ischemia/reperfusion (I/R) during the very early phase of reperfusion protect the myocardium just as pre-conditioning does, and named this phenomenon ischemic post-conditioning.(Zhao et al., 2003) Subsequent studies have demonstrated that ischemic post-conditioning reduces infarct size and the incidence of arrhythmia in several animal models. (Kin et al., 2004; Yang et al., 2004; Heusch et al., 2006; Mykytenko et al., 2007) This cardioprotective effect can also be achieved by pharmacological stimuli. Many studies have shown that volatile anesthetics attenuate the myocardial injury against I/R (or anesthetic post-conditioning).(Kersten et al., 1997; Feng et al., 2006; Pravdic et al., 2010; Ge et al., 2010) Multiple kinases, including PI3K–Akt,(Feng et al., 2006) extracellular signal-regulated kinase (ERK) 1/2,(Yao et al., 2010) GSK 3β,(Lemoine et al., 2010) eNOS,(Ge et al., 2010) and BCl-2(Wang et al., 2006) are involved in signal transduction pathways of anesthetic post-conditioning.

Although a great deal of experimental evidence has indicated that volatile anesthetics administered at the onset of reperfusion protect the heart against ischemia-reperfusion injury, the cardioprotective effect of volatile anesthetics in clinical situations is not apparent.(Julier et al., 2003; Lee et al., 2006; Piriou et al., 2007; De Hert et al., 2009) The poor translation of experimental findings to clinical contexts may be due to the characteristics of the target population; while most experimental studies have been performed in healthy young animals, surgical patients are often diseased and elderly. The effects of aging on the cardioprotective effect of pre- and post-conditioning by variable stimuli are controversial. Although some studies found that cardioprotection was preserved in aged animals,(Dai et al., 2009) others showed that aging was associated with a loss of cardioprotection by pre- or post-conditioning.(Sniecinski and Liu, 2004; Przyklenk et al., 2008)

We investigated the cardioprotective effect of isoflurane administered at the onset of reperfusion in senescent rat in vivo and the activation of reperfusion injury salvage kinase (RISK) pathways, including ERK1/2, Akt, and GSK 3β, to address a possible mechanism underlying age-related differences.

Ⅱ. MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine, and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, National Academy Press, Washington DC). The Association for Assessment and Accreditation of Laboratory Animal Care International accredits Yonsei University College of Medicine.

Surgical instrumentation

Male Wistar rats aged 3–5 months and 20–24 months were obtained and housed in the Division of Laboratory Animal Resources until the day of the experiment. These ages correspond to approximately 20–30 and 65–75 human years, respectively.(Sniecinski and Liu, 2004) Rats were anesthetised with sodium thiobutabarbital (100–150 mg/kg, i.p.) and received additional doses of thiobutabarbital (25–50 mg/kg) to ensure that pedal and palpebral reflexes were absent throughout the experimental protocol. Rats were instrumented for the measurement of systemic hemodynamics as previously described.(Ludwig et al., 2003) Briefly, heparin-filled catheters were inserted into the right jugular vein and the right carotid artery for fluid or drug administration and measurement of arterial blood pressure, respectively. A tracheostomy was performed by intubating the trachea with a cannula connected to Harvard rodent ventilator Model 683 (Harvard Apparatus, Inc., MA, USA), and the lungs were ventilated with 2–3 cmH_2O positive end-expiratory pressure and an air–oxygen mixture (fractional inspired oxygen concentration = 0.5). Ventilatory frequency was adjusted to maintain Pco_2 at 35 ± 5 mmHg. Body temperature was maintained at 37 ± 5 °C using a heating pad and radiant warmer. A thoracotomy was performed in the left fifth intercostal space, and the pericardium was opened. A 6-0 Prolene ligature was placed around the proximal left descending coronary artery and vein in the area immediately below the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. Coronary artery occlusion was produced by clamping the snare onto the epicardial surface with a hemostat, and was confirmed by the appearance of epicardial cyanosis and ST

elevation on electrocardiography (EKG). Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperaemia. Hemodynamic data were continuously recorded on a data acquisition system (Power Lab; AD Instruments, Colorado Springs, CO, USA).

Experimental protocol for infarct size determination and western immunoblotting

The experimental design is illustrated in Fig. 1. Baseline hemodynamic and arterial blood gas tension was recorded 30 min after instrumentation. All rats underwent 30 min of coronary artery occlusion, followed by 2 h of reperfusion. In four separate groups, rats (n = 6–7 per group) of similar age were randomly assigned to receive isoflurane 1.0 MAC for 3 min before and 2 min after reperfusion (isoflurane group) or not (control group). At the end of each experiment, hearts were excised for the determination of myocardial infarct size.

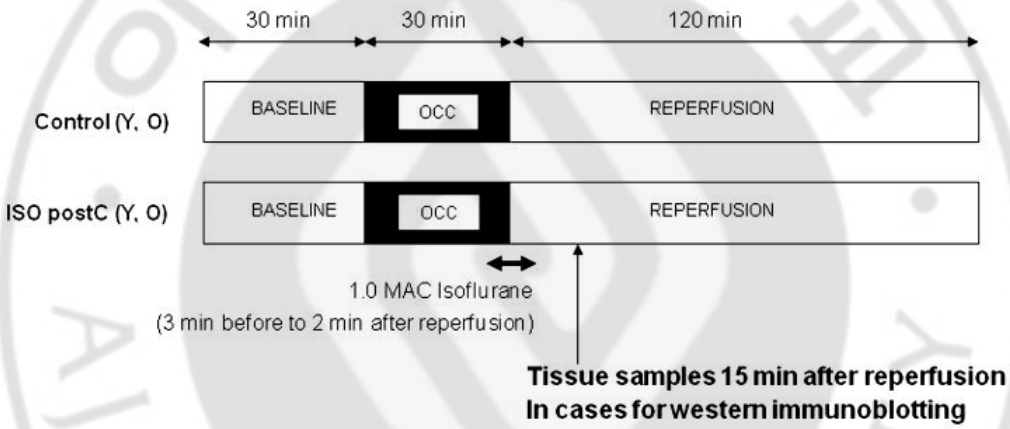

Fig. 1. Schematic diagram depicting the experimental protocol. Control, control group; ISO postC, isoflurane post-conditioning for 5 min; Y, young rats; O, old rats; OCC, occlusion; MAC, minimum alveolar concentration.

For western immunoblotting, rats underwent 30 min of coronary artery occlusion, followed by reperfusion. In four additional groups of rats (n = 5 in each group), left ventricular (LV) samples were collected at 15 min after reperfusion (Fig. 1).

Determination of myocardial infarct size

Myocardial infarct size was measured as previously described.(Ludwig et al., 2003) Briefly, the coronary artery was reoccluded after 2-h reperfusion. Patent blue dye was administered IV to stain the normal region of the LV, and the heart was rapidly excised. The LV was cut into five or six cross-sectional pieces of 2-mm thickness. The non-stained left ventricle area at risk (AAR) was separated from the blue-stained LV normal zone and incubated at 37°C for 15 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH 7.4. After overnight fixation in 10% formaldehyde, infarcted tissue (non-stained) and non-infarcted area (red-stained) within the AAR were carefully separated under a dissecting microscope and weighed. Infarct size was expressed as a percentage of the left ventricular AAR.

Western immunoblotting

LV samples were collected at the times indicated on Fig. 1, and were immediately frozen in liquid nitrogen and stored at −70°C for subsequent analysis. The frozen tissue samples were pulverised and homogenised in ice-cold buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, complete protease inhibitor cocktails (one tablet per 10 Mℓ; Roche Diagnostics Corporation, Indianapolis, IN, USA), and phosphatase inhibitor cocktail set II (EMD Biosciences, Darmstadt, Germany). The homogenate was centrifuged at 10,000 g for 20 min and 13,000 g for 15 min at 4°C. Protein concentration was determined using a modified Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. Protein samples were prepared by mixing with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) and heated at 97°C for 5 min. Equivalent amounts (30 μg) of protein were loaded and separated on 10% SDS-polyacrylamide gels, and then electrophoretically transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline (TBS) containing 10% milk and 0.1% Tween 20 for 1 h at room temperature, membranes were incubated overnight at 4°C with the following antibodies: mouse monoclonal p42 MAP kinase (3A7), 1 : 1000 dilution in 5% nonfat dry milk; rabbit monoclonal phospho-p44/42 MAP kinase (at Thr202/Tyr204), 1 : 1000 dilution in 5% nonfat dry milk; rabbit monoclonal Akt, 1 : 1000 dilution in 5% bovine serum albumin (BSA); rabbit monoclonal phospho-Akt (at Ser473), 1 : 1000 dilution in 5% BSA; rabbit monoclonal GSK-3β (27C10), 1 : 1000 dilution in 5% BSA; and rabbit

monoclonal phospho-GSK-3β (at Ser9), 1 : 1000 dilution in 5% BSA. All antibodies were manufactured by Cell Signaling Technology (Beverly, MA, USA). The membranes were washed five times for 5 min, and subsequently incubated for 1 h in 5% nonfat dry milk in TBST containing either a goat anti-mouse (Bio-Rad Laboratories, Hercules, CA, USA) or donkey anti-rabbit (GE Healthcare UK Limited, Buckinghamshire, UK) immunoglobulin G conjugated to horseradish peroxidase. Immunoreactive bands were visualised by chemiluminescence detected on X-ray film (GE Healthcare UK Limited) using the enhanced chemiluminescence substrate system (GE Healthcare UK Limited). The optical density of each band was determined using UN-SCAN-IT (Silk Scientific, Inc., Orem, UT, USA) and normalised against background density for each membrane. The results are presented as the ratios of phosphorylated to total protein. Values are expressed as percentages compared with controls.

Statistical analysis

Statistical analysis of data within and between groups was performed with multiple repeated measures ANOVA followed by Student Newman–Keuls tests. Changes were considered statistically significant when P values were less than 0.05. All values are expressed as mean ± standard deviation (SD).

Ⅲ. RESULTS

Thirty-two rats were instrumented to obtain six to seven successful measurements per group of myocardial infarct size. Of the 32 rats, three old rats were excluded because intractable ventricular fibrillation occurred during coronary artery occlusion, one old rat was excluded because of technical difficulties during instrumentation, and one young rat with AAR < 30% was also excluded. For western blotting, 22 rats were instrumented to obtain five successful LV samples. One old rat was excluded due to intractable ventricular fibrillation during the coronary artery occlusion, and one young rat was excluded due to the absence of EKG changes after coronary artery occlusion.

Systemic hemodynamics

There were no differences in baseline hemodynamics among the groups (Table 1). A decrease in mean arterial pressure was observed during reperfusion in all groups. Rate-pressure product in the young control group was decreased during reperfusion. There were no differences in hemodynamics among the groups before, during, or after occlusion of left anterior descending coronary artery.

Table 1. Hemodynamic data

	young		old	
	control (n=7)	isoflurane (n=7)	control (n=6)	isoflurane (n=7)
Heart rate (beats/min)				
pre-Occ	399 ± 58	402 ± 59	316 ± 50	302 ± 55
Occ 15′	451 ± 87*	433 ± 49	353 ± 34*	317 ± 75
Iso 3′		358 ± 46		258 ± 82
Rep 60′	395 ± 28	420 ± 34	347 ± 44	319 ± 23
Rep 120′	403 ± 36	449 ± 68	329 ± 50	320 ± 40
Mean arterial pressure (mmHg)				
pre-Occ	111 ± 13	97 ± 21	101 ± 21	106 ± 16
Occ 15′	108 ± 20	93 ± 21	105 ± 18	95 ± 19

Iso 3′		54 ± 8‡		65 ± 28‡
Rep 60′	85 ± 15‡	94 ± 16	92 ± 14	83 ± 21*
Rep 120′	84 ± 14‡	82 ± 17*	76 ± 14†	79 ± 17†

Values are expressed as mean ± standard deviation. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs. pre-Occ in each group. Occ, occlusion of LAD; Iso, isoflurane; Rep, reperfusion.

Myocardial infarct size

Body weights and LV weights were similar between the young control and young isoflurane groups, and between the old control and old isoflurane groups (Table 2). The ratios of AAR were also similar among groups. A brief administration of isoflurane 3 min before and 2 min into early reperfusion reduced infarct size (58 ± 10% of left ventricular AAR, mean ± SD) compared with control (68 ± 4%) in young rats, whereas isoflurane did not cause this reduction in old rats (56 ± 8% in isoflurane group and 56 ± 10% in control group) (Fig. 2).

Table 2. Weights and area at risk

	young		old	
	control (n=7)	isoflurane (n=7)	control (n=6)	isoflurane (n=7)
body weight (g)	350 ± 35	327 ± 40	623 ± 84*	672 ± 41*
LV weight (mg)	595 ± 77	516 ± 69	1014 ± 110*	928 ± 116*
Area at risk (%)	43 ± 7	44 ± 6	43 ± 4	41 ± 7

Values are expressed as mean ± standard deviation. *$P < 0.001$ vs. young control group. LV, left ventricle.

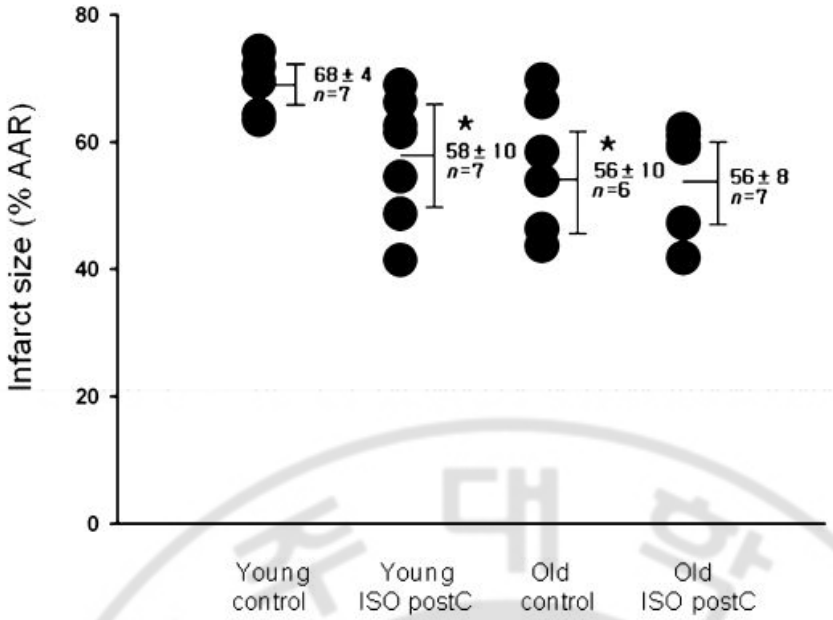

Fig. 2. Myocardial infarct size expressed as a percentage of the left ventricular area at risk in rats receiving isoflurane 3 min before and 2 min after reperfusion (ISO postC) or not (control). All data are expressed as the mean ± standard deviation. *P < 0.05 vs. young control. The old ISO postC group was compared with the young ISO post-C and old control groups, but there were no significant differences. AAR, area at risk; ISO postC, isoflurane post-conditioning.

Phosphorylation of ERK, Akt, and GSK 3β (Fig. 3)

The phosphorylation levels of ERK1/2 (Fig. 3A), Akt (Fig. 3B), and GSK 3β (Fig. 3C) were increased in the young ISO postC group but not in the old ISO postC group compared with the control group in age-matched controls. There were no differences in phosphorylation of ERK1/2, Akt, and GSK 3β between the young control and old control groups.

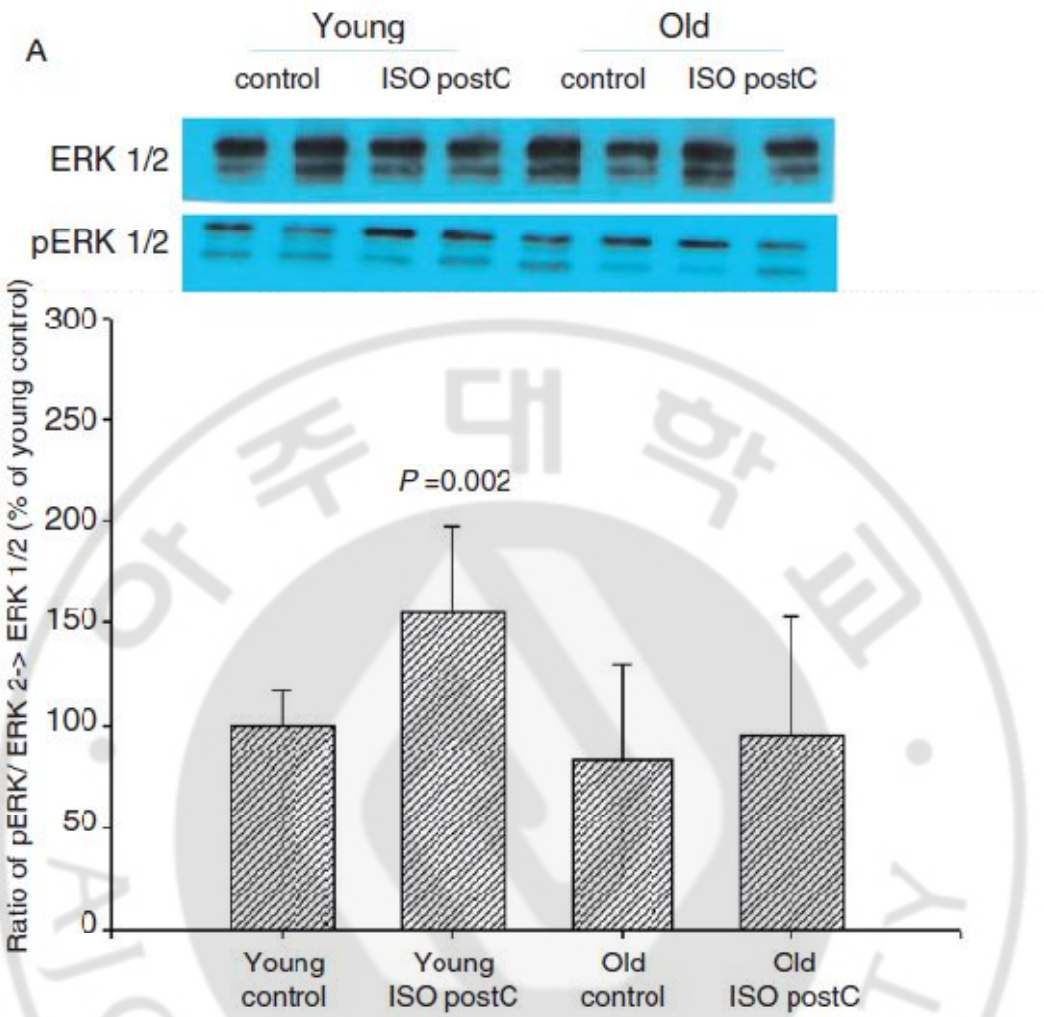

Fig. 3. Western blot analysis of (A) ERK1/2 and p-ERK1/2, (B) Akt and p-Akt, and (C) GSK 3β and p-GSK 3β after 30 min of reperfusion in rats (n = 5 for each group) receiving 0.9% saline (control) or isoflurane post-conditioning (ISO postC) 3 min before and 2 min after reperfusion. Each signal was quantified by scanning densitometry. *P < 0.05 vs. age-matched control group.

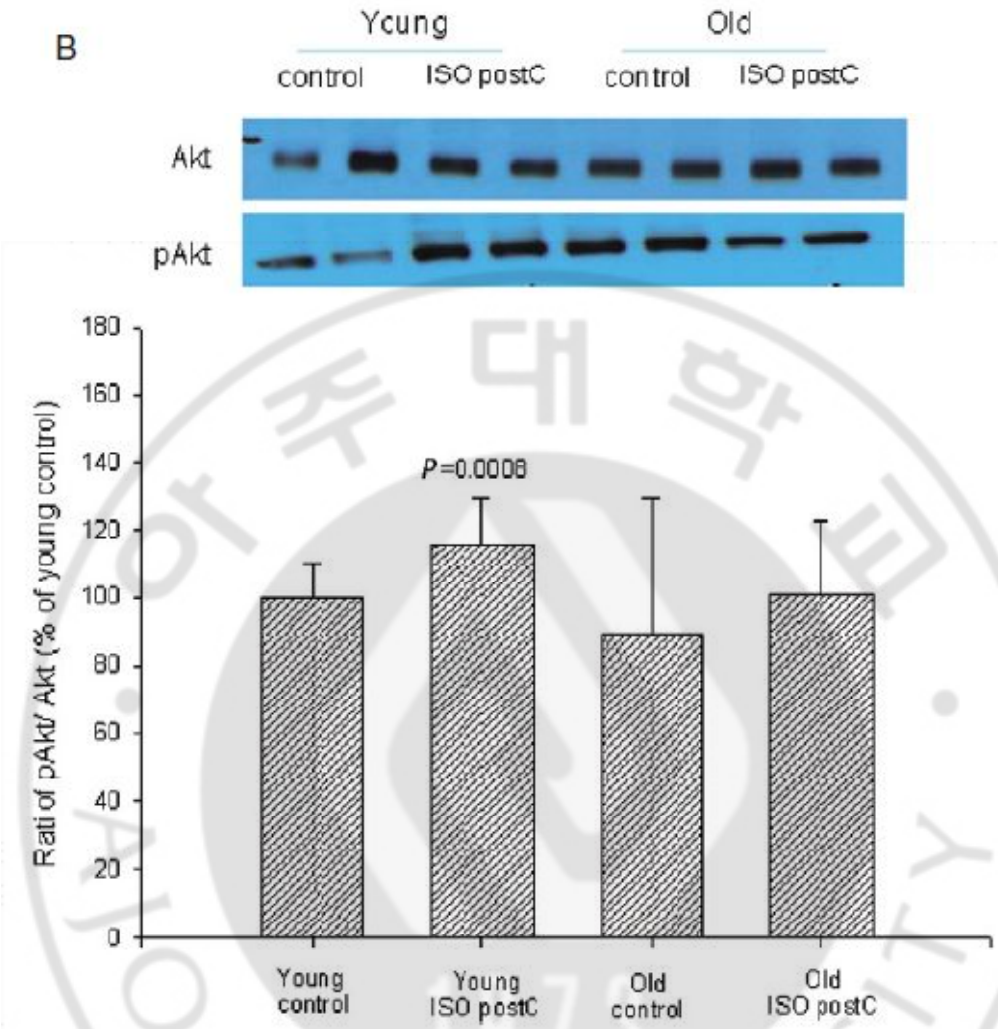

Fig. 3. Continued

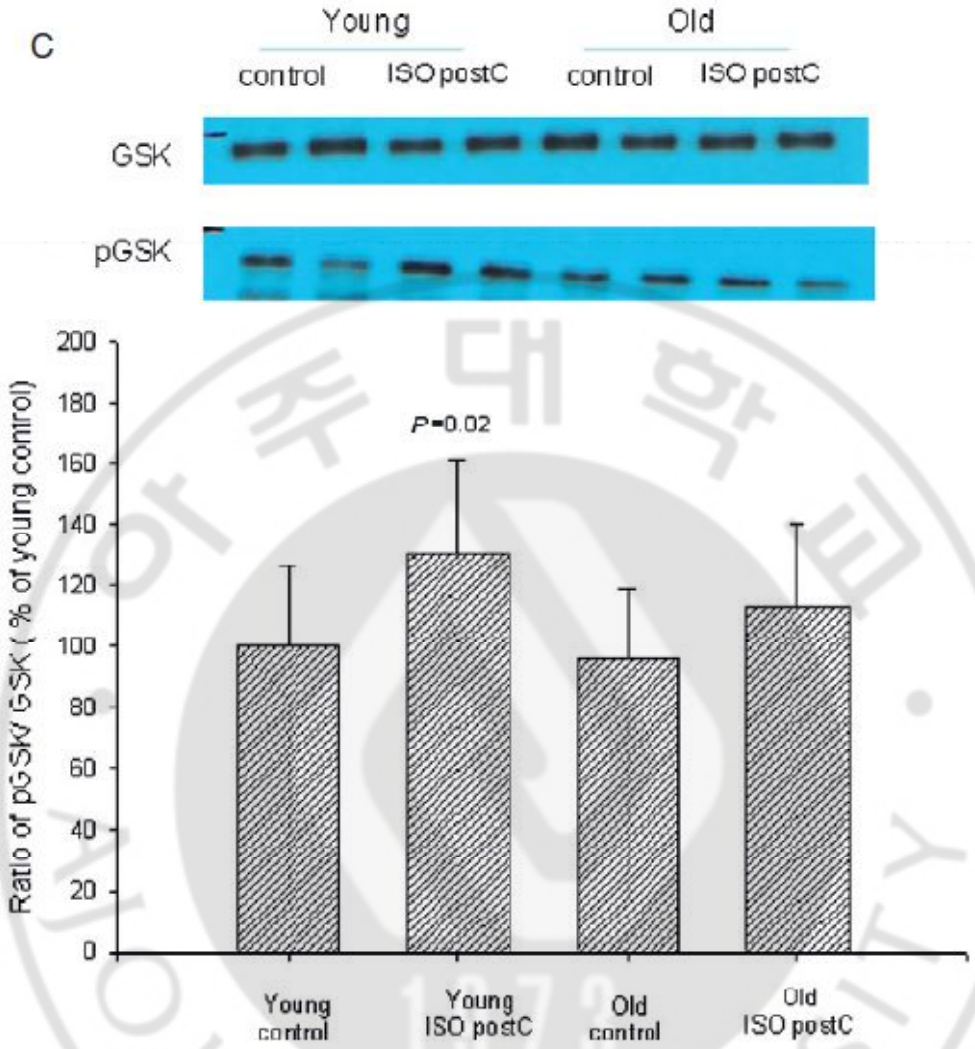

Fig. 3. Continued

Ⅳ. DISCUSSION

In our in vivo study, brief administration of isoflurane 3 min before and 2 min into early reperfusion reduced myocardial infarct size compared with the respective control group in young rats. In contrast, there was no reduction in myocardial infarct size in old rats compared with the age-matched control group. Furthermore, the phosphorylation of Akt, GSK 3β, and ERK were elevated by isoflurane administration in young rats but not in old rats, suggesting that failure to activate the RISK pathway may be associated with the inability of isoflurane-induced post-conditioning to protect the senescent heart.

Cardioprotection by pharmacologic stimuli, compared with ischemic stimuli, is of great practical interest because it does not require the induction of ischemia, which could greatly exacerbate symptoms and reduce cardiac reserve. In addition, post-conditioning is more practical for clinical applications since interventions can be timed to the opening of the occluded vessel. This study provides the first evidence that anesthetic post-conditioning is not maintained in aged myocardium, probably due to failure to activate pro-survival kinases.

Our results demonstrate that cardioprotection by anesthetic post-conditioning is attenuated in the aged myocardium, similar to the attenuated response to anesthetic pre-conditioning seen in the aged myocardium.(Nguyen et al., 2008; Zhu et al., 2010) It was previously shown that isoflurane pre-conditioning did not reduce the fall in NAD (+) levels or infarct size in 20- to 24-month-old male Fisher 344 rats.(Nguyen et al., 2008; Zhu et al., 2010) Some researchers have reported that cardioprotection by ischemic or pharmacologic stimuli is maintained in old animals.(Przyklenk et al., 2001; McCully et al., 2006) However, considering the maximal lifespan (13 years) of rabbits, the animals younger than 4 years, used in those experiments, might actually represent the middle-aged rather than the elderly. Clinical data also support loss of cardioprotection with aging. The presence of angina before an acute myocardial protection, a clinical equivalent to an experimental ischemic pre-conditioning, did not improve in-hospital outcomes in elderly (> 65 years old) compared with younger patients (< 65 years old).(Abete et al., 1997)

Western blotting showed that phosphorylation of RISK elements was not elevated by isoflurane post-conditioning in old rats, while it was elevated in young rats. Anesthetic post-

conditioning with volatile anesthetics shares a similar pathway with anesthetic pre-conditioning, although those mechanisms are not certain. RISK pathway, a group of pro-survival kinases, plays a critical role in cardioprotection when activated at reperfusion. Use of this pathway is considered common in cardioprotection by ischemic or pharmacologic stimuli, and seems to converge in the mitochondrial permeability transition pore. The main members of the RISK pathway are the PI3K–Akt and ERK1/2 kinase cascades.(Hausenloy and Yellon, 2007) In isolated rat hearts, improved cardiac function and decreased infarct size by anesthetic post-conditioning were reported to occur through the Akt/GSK 3β pathway.(Feng et al., 2005; Feng et al., 2006; Fang et al., 2010; Inamura et al., 2010) Activation of ERK was also found to be related to cardioprotection by anesthetic post-conditioning.(Chen et al., 2008) Our results showing that RISK-associated pathways were activated by isoflurane post-conditioning in young rats are consistent with those of previous studies. However, in aged rat myocardium, RISK pathways were not activated by isoflurane post-conditioning, which might be causal in the attenuation of infarct size reduction in old rats.

The aged myocardium is susceptible to ischemia-reperfusion injury. However, reports on the infarct size in the aged myocardium are inconsistent. Most studies reported that infarct sizes are not different between young and old animals,(Nguyen et al., 2008) while others reported that infarct size is larger in old mice.(Azhar et al., 1999) In our study, infarct size in aged myocardium was found to be smaller than that in young rats. The difference in the size of total LV mass might be difficult of direct comparison between two different age groups.

The expression or activation of RISK elements at baseline may also be changed with aging. Some reports have shown that the baseline levels of protein kinases were altered, although the reported results have not been consistent. Zhu et al. showed that the increase in phosphorylated GSK 3β by isoflurane pre-conditioning was impaired in the aged myocardium, even though the baseline phosphorylated GSK 3β was elevated in this same myocardium.(Zhu et al., 2010) In contrast, our results showed that the baseline levels of phosphorylated RISK elements were not different between old and young rats.

There are some limitations in our study. First, the same dosage of isoflurane was used in both age groups even though the MAC value declines with age. Second, only one dose of isoflurane was used in old rats. Some research reported that a greater stimuli could perhaps

overcome the impairment in old myocardium and result in downstream effects similar to those seen in young myocardium,(Schulz et al., 1998) but others reported that enhanced stimuli were unable to overcome these changes.(Peart and Headrick, 2009) Last, we did not stimulate RISK pathways directly in aged myocardium.

Ⅴ. CONCLUSION

In conclusion, our results showed that an attenuation of isoflurane-induced post-conditioning to reduce myocardial infarct size in aged rats was paralleled by a reduction in phosphorylation of Akt, ERK, and GSK 3β, providing evidence that aged myocardium is refractory to reduction in infarct size by isoflurane post-conditioning, possibly due to impairment in phosphorylation of RISK pathway members.

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– 국문 요약 –

노화된 심근에서 isoflurane 후조건화의 보호효과 감소에 대한 연구: Reperfusion Injury Salvage Kinase 활성화의 역할

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본 연구에서는 고령 쥐에서 isoflurane 을 심근의 재관류 시기에 투여하였을 때 흡입마취제의 심근보호 효과를 젊은 쥐와 비교하여 알아보고자 하였다. 또한 이러한 차이를 나타내는 이유로 reperfusion injury salvage kinase (RISK) pathway 의 활성화 정도를 측정하여 비교해 보고자 하였다.

수컷 Wistar 쥐를 나이에 따라 3-5 개월 된 젊은 쥐와 20-24 개월 된 고령 쥐로 나누고 각각을 다시 재관류 시작 3 분 전부터 2 분 후까지 5 분간 isoflurane (1 최소 폐포 농도)을 투여 받는 그룹(ISO popstC 군)과 그렇지 않은 그룹(대조군)으로 무작위로 나누어 실험을 진행하였다. 모든 그룹에서 관상동맥의 결찰은 30 분간 유지하였고 이후 2 시간 동안 재관류를 하였다. 재관류 시작 후 15 분이 경과하였을 때 얻은 심근 조직으로 western blot analysis 를 시행하여 ERK1/2, Akt, GSK3β 의 인산화 정도를 측정하였다.

젊은 쥐에서는 isoflurane 을 투여한 군에서 심근 경색의 정도가 대조군에 비하여 감소하여 나타났으나 (ISO postC 군; 56±8% of left ventricle area at risk, 대조군; 68±4%, 평균±표준편차) 고령 쥐에서는 유의한 차이를 보이지 않았다 (ISO postC 군; 56±8%, 대조군; 56±10%). Isoflurane 의 투여 시 젊은 쥐에서는 ERK1/2, Akt, GSK3β 의 인산화 정도가 증가하였으나 고령 쥐에서는 차이가 없었다.

Isoflurane 의 후조건화 효과가 젊은 쥐에서는 유의하게 나타났으나 고령 쥐에서는 나타나지 않았고 이것은 고령 쥐에서 RISK pathway 가 활성화 되지 못한 것과 연관이 있을 것으로 보인다.

핵심어: 심근, 후조건화, isoflurane, RISK pathway