

Repeated or Prolonged Isoflurane Exposure Reduces Mitochondrial Oxidizing Effects

Shinji Kohro, M.D., Ph.D.,* Quinn H. Hogan, M.D.,† Yuri Nakae, M.D.,* Michiaki Yamakage, M.D., Ph.D.,‡
Zeljko J. Bosnjak, Ph.D.§

VOLATILE anesthetics are well known to have cardioprotective effects similar to those of ischemic preconditioning,¹⁻¹¹ but the mechanisms remain unclear. Recent evidence indicates that the protective effects of K_{ATP} channel openers may be due to action on mitochondrial K_{ATP} channels.¹²⁻¹⁶ We have previously reported that 15 min of volatile anesthetic exposure induces mitochondrial flavoprotein oxidation *via* mitochondrial K_{ATP} channel activation,¹⁷ which may contribute to anesthetic-induced preconditioning. Volatile anesthetic exposure produces a memory period after exposure during which damage from ischemia is diminished, even though the anesthetic is no longer being administered.⁸ We designed the present study to examine time factors that may influence mitochondrial oxidation in anesthetic-induced preconditioning using mitochondrial flavoprotein fluorescence (MFF) as an endogenous reporter of mitochondrial redox state.^{13,14,18}

Methods

US National Institutes of Health Standards (NIH Publication #95-23, Revised 1996) and our Institutional Animal Care Committee policies were followed.

Cell Isolation

Cardiac myocytes were isolated from ventricles of guinea pigs (200-300 g) as previously described.¹⁷ Briefly, during pentobarbital anesthesia, the hearts were quickly excised, mounted on a Langendorff apparatus, and perfused *via* the aorta with an oxygenated enzyme solution containing Joklik minimum essential medium, 0.4 mg/ml collagenase (Gibco, Grand Island, NY) and 0.17 mg/ml protease (Sigma, St. Louis, MO). The digested ventricular tissue was chopped coarsely and

shaken in a water bath for further dispersion. The cells were filtered, centrifuged, and washed. Rod-shaped cells with clear borders and striations were used within 8 h of isolation.

Flavoprotein Fluorescence Measurements

Cells were superfused with modified Tyrode solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, and 2 CaCl₂ (pH 7.4 with NaOH) at 21°C. MFF¹²⁻¹⁴ was excited every 30 s with light from a xenon laser band-pass filtered to 488 ± 20 nm, and emission was recorded through a 515-nm long pass filter. Relative fluorescence was averaged during excitation, and fluorescence intensity was expressed as arbitrary units (au; range, 0-256). At the end of each protocol, the maximum MFF was determined by administration of dinitrophenol (100 μM), and the minimum MFF was determined by administration of cyanide (4 mM).

Experimental Protocols

From previously published data, 15 min of inhalational anesthetic exposure was shown to be effective for preconditioning *in vitro*⁹ and *in vivo*.^{2,7} Therefore, we used a 15-min period of isoflurane (Abbott Laboratories, Chicago, IL) exposure that was preceded by 10 min of equilibration with glucose-free Tyrode buffer (fig. 1). For a repeated exposure protocol, isoflurane was administered three separate times for 15 min interposed by 10-min washout intervals. For a sustained exposure protocol, isoflurane was administered for 45 min followed by 10 min of washout. To test the effect of a long delay interval between repeated exposures, a 15-min exposure of isoflurane was followed by 45 min of washout and then a second exposure lasting 15 min. In each protocol, two sets of isoflurane concentrations were used, which were measured by gas chromatography.

Statistical Analysis

Data are presented as mean ± SD, and the number of cells is shown as n. Analysis of variance for repeated measurements and Scheffé tests were used to verify differences in fluorescence data.

Results

With repeated isoflurane administration (figs. 2A and B), second and third exposures produced a peak MFF response at the beginning of each exposure rather than

* Research Fellow, † Professor, Departments of Anesthesiology and Anesthesiology Research, § Professor, Departments of Anesthesiology, Anesthesiology Research, and Physiology, Medical College of Wisconsin. ‡ Assistant Professor, Department of Anesthesiology, Sapporo Medical University School of Medicine.

Received from the Departments of Anesthesiology, Anesthesiology Research, and Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin; and the Department of Anesthesiology, Sapporo Medical University School of Medicine, Sapporo, Japan. Submitted for publication April 3, 2002. Accepted for publication August 16, 2002. Supported in part by grant No. HL34708 from the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, and by the Department of Anesthesiology, Sapporo Medical University, Sapporo, Japan (Prof. Akiyoshi Namiki, M.D., Ph.D.).

Address reprint requests to Dr. Hogan: Anesthesiology Research, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226. Address electronic mail to: qhogan@mcw.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

A.	Baseline	ISO washout		ISO washout		ISO
		15	10	15	10	15
B.	Baseline	ISO			washout	
		45			10	
C.	Baseline	ISO	washout			ISO
		15	45			15

Fig. 1. Time line of the experimental protocols. Baseline indicates a period of no experimental intervention. (A) Repeated exposure: 15 min of isoflurane (ISO) exposure and 10 min of washout with glucose-free Tyrode buffer were repeated three times. (B) Long-term exposure: 45 min of isoflurane exposure followed by 10 min of washout. (C) Long-interval, repeated exposure: 15 min of exposure was followed by a 15-min second exposure after 45 min of washout.

a sustained increase to a peak, as seen during the first exposure (fig. 2A). MFF concentrations at the end of each exposure were sequentially decreased both during high-concentration isoflurane exposure (32.1 ± 4.1 , 30.5 ± 4.5 , and 27.8 ± 2.6 au for the first, second, and third exposure, respectively; $P < 0.0001$) and low-concentration isoflurane exposure (26.6 ± 3.2 , 23.0 ± 3.1 , and 18.7 ± 1.9 au for the first, second, and third exposure, respectively; $P < 0.0001$; fig. 2B). During administration of low-concentration isoflurane, MFF at the end of the third exposure was less than the baseline concentration (22.6 ± 1.5 vs. 18.7 ± 1.9 au for baseline vs. third exposure, $P < 0.01$; fig. 2B). During this protocol, isoflurane concentrations were 1.5 ± 0.3 mM (high) and 0.8 ± 0.3 mM (low).

At the beginning of sustained (45-min) isoflurane exposure, high-concentration isoflurane showed stronger effects on MFF than low-concentration isoflurane (22.4 ± 2.1 vs. 31.6 ± 2.5 au for low-concentration vs. high-concentration isoflurane, $P < 0.01$; figs. 3A and B). By the end of the exposure, a decreasing MFF during high-concentration isoflurane exposure resulted in concentrations lower than those during low-concentration isoflurane exposure (29.2 ± 2.9 vs. 23.7 ± 3.9 au for low-concentration vs. high-concentration isoflurane exposure, $P < 0.01$). After washout, although MFF in high-concentration isoflurane returned to the same concentration as baseline (23.3 ± 2.2 vs. 23.7 ± 3.9 au for baseline vs. washout, $P = 0.81$), the MFF during low-concentration isoflurane exposure was still higher than baseline (22.4 ± 2.1 vs. 29.2 ± 2.9 au for baseline vs. washout, $P < 0.01$). In this protocol, measured isoflurane concentrations were 1.5 ± 0.1 mM (high) and 0.9 ± 0.1 mM (low).

In the third protocol, with delayed reexposure after 45 min of washout, MFF of cells previously exposed to low-concentration or high-concentration isoflurane were both below original baseline (low-concentration isoflurane: 21.1 ± 2.2 vs. 18.7 ± 2.1 au for baseline vs. after washout, $P < 0.05$; high-concentration isoflurane: 22.4 ± 1.4 vs. 20.6 ± 1.6 au for baseline vs. after washout, $P < 0.05$; figs. 4A and B). A second exposure of isoflurane did not increase MFF in either group (low-

concentration isoflurane: 18.7 ± 2.1 vs. 19.2 ± 3.3 for after washout vs. second exposure, $P = 0.73$; high-concentration isoflurane: 20.6 ± 1.6 vs. 22.1 ± 2.2 for after washout vs. second exposure, $P = 0.14$). In this protocol, measured isoflurane concentrations were 1.7 ± 0.2 mM (high) and 0.8 ± 0.1 mM (low).

Discussion

The principal findings of these experiments are that alterations in mitochondrial redox state induced by isoflurane are dependent on the concentration and temporal pattern of exposure. Whereas brief exposure to volatile anesthetic results in mitochondrial flavoprotein oxidation, sustained exposure, especially to high concentrations of isoflurane, leads to depression of oxidation and failure to respond to subsequent anesthetic exposure.

It is unknown whether mitochondrial oxidation or reduction is advantageous for myocardial protection during ischemia. Ischemic preconditioning reduces myocardial oxygen consumption during ischemia,¹⁹ which may be accounted for by decreased contractile activity and by depression of mitochondrial respiration by nitric oxide. It is therefore possible, despite our earlier findings of an initial volatile anesthetic oxidation response,¹⁷ that isoflurane ultimately reduces mitochondrial oxidation and myocardial oxygen consumption, thereby inducing myocardial protection during ischemia.

From the present data on repeated isoflurane exposure, it is evident that the first exposure activates processes that change the sensitivity for subsequent mitochondrial oxidation and reduces the oxidizing effect of subsequent isoflurane exposures. This negative effect persists after a 45-min washout interval. The depressed resting concentrations of oxidation even 45 min after isoflurane exposure provide further evidence supporting a prolonged decreased oxidation in cells previously exposed to isoflurane.

We observed that, unlike exposure to high-concentration isoflurane, 45 min of exposure to low-concentration isoflurane continues to increase the oxidation concen-

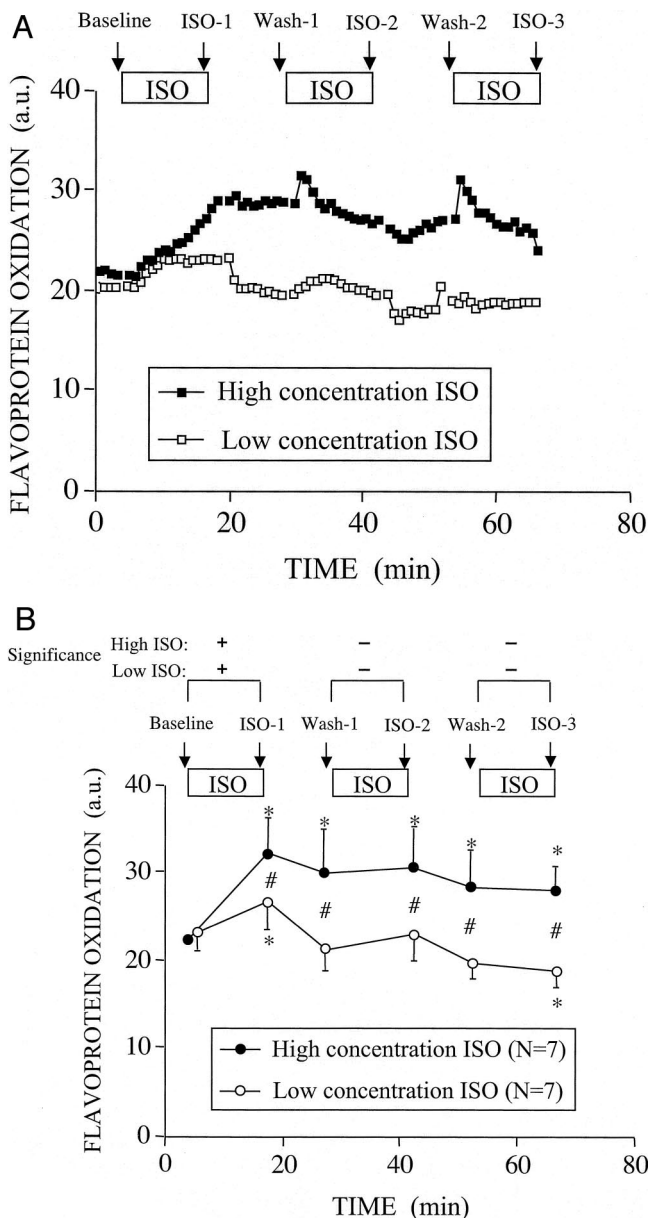


Fig. 2. (A) Typical raw traces of mitochondrial flavoprotein fluorescence (MFF) in repeated isoflurane (ISO) exposures. (B) Mean (\pm SD) MFF in repeated isoflurane exposures. Data were analyzed at the end of each isoflurane exposure and washout (baseline, first isoflurane exposure [ISO-1], first washout [Wash-1], second isoflurane exposure [ISO-2], second washout [Wash-2], and third isoflurane exposure [ISO-3]). Concentrations of isoflurane were 1.5 ± 0.3 mm (high) and 0.8 ± 0.3 mm (low). * $P < 0.05$ versus baseline; # $P < 0.05$ high versus low isoflurane concentration; + $P < 0.05$ for first, second, or third isoflurane exposure versus immediately preceding baseline or washout, as determined by Scheffé test after analysis of variance for repeated measures. a.u. = arbitrary units.

tration. We speculate that there is a difference in threshold between oxidizing and suppressive pathways for isoflurane-induced changes. We are not sure why repeated exposure to the same low concentration of isoflurane shows a dominance of the suppressive effect on oxidation.

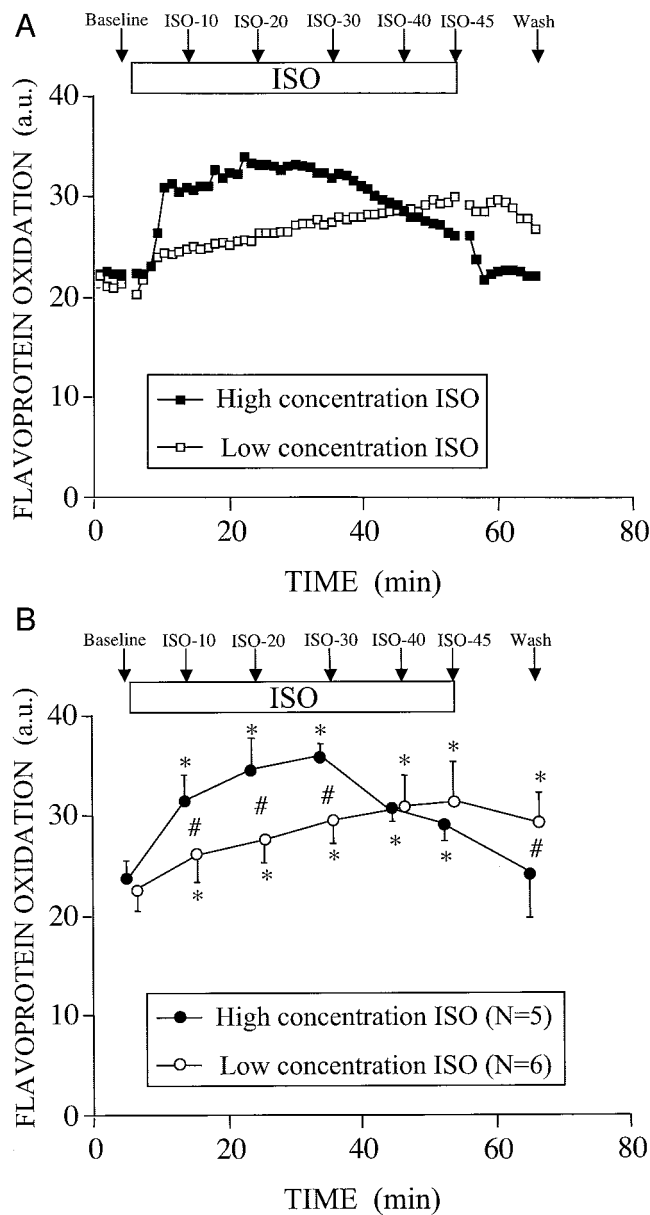


Fig. 3. (A) Typical raw traces of mitochondrial flavoprotein fluorescence (MFF) in 45-min isoflurane (ISO) exposures. (B) Mean (\pm SD) MFF in 45-min isoflurane exposures. Data were analyzed before exposure to isoflurane and at baseline; 10 (ISO-10), 20 (ISO-20), 30 (ISO-30), 40 (ISO-40), and 45 min (ISO 45) during isoflurane exposure; and after washout (Wash). Isoflurane concentrations were 1.5 ± 0.1 mm (high) and 0.9 ± 0.1 mm (low). * $P < 0.05$ versus baseline; # $P < 0.05$ high versus low isoflurane concentration, as determined by Scheffé test after analysis of variance for repeated measures. a.u. = arbitrary units.

Inhibition of the respiratory chain or addition of uncouplers of oxidative phosphorylation can both limit the extent of enzyme release in the intact heart and prevent the onset of irreversible morphologic changes in isolated myocytes.²⁰ Volatile anesthetics are known to have mitochondrial uncoupling effects that may reduce the damage to myocytes. Both of these facts are compatible with our hypothesis that the beneficial preconditioning ef-

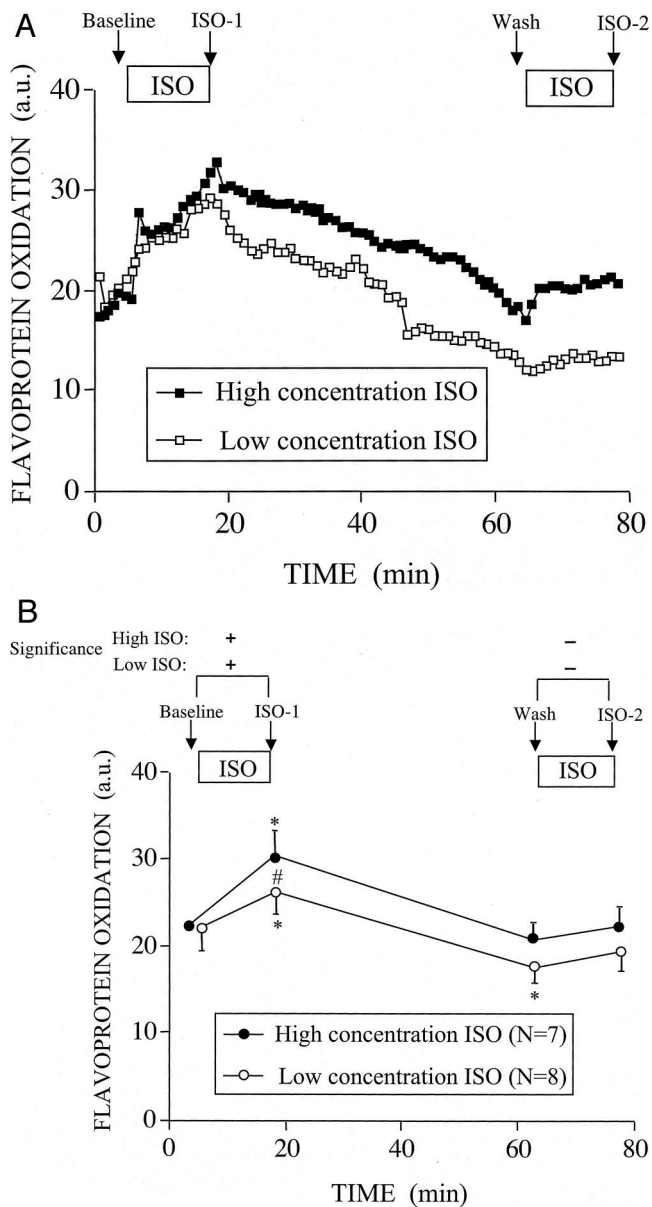


Fig. 4. (A) Typical raw traces of mitochondrial flavoprotein fluorescence MFF in repeated isoflurane (ISO) exposure following a 45-min interval. (B) Mean (\pm SD) MFF in repeated and long-interval isoflurane exposures. Data were analyzed at each end of isoflurane exposures and washout (baseline, first isoflurane exposure [ISO-1], washout [Wash], and second isoflurane exposure [ISO-2]). Isoflurane concentrations were 1.7 ± 0.2 mM (high) and 0.8 ± 0.1 mM (low). * $P < 0.05$ versus baseline; # $P < 0.05$ high versus low isoflurane concentration; + $P < 0.05$ for first or second isoflurane exposure versus immediately preceding baseline or washout, as determined by Scheffé test after analysis of variance for repeated measures. a.u. = arbitrary units.

effects of volatile anesthetic exposure are due to subsequent inhibition of myocyte oxidation. One possible intracellular pathway mediating these effects may be the generation of free radicals that trigger ischemic preconditioning memory and activation of kinases.²¹

Because our experiments examined isolated, nonbeating myocytes at room temperature, we cannot be certain that the mitochondrial redox state in the beating and warm heart responds in the same fashion. Nonetheless, our observation of the contrasting response to a single exposure compared with repeated or prolonged exposure indicates unexpected complexity of isoflurane effects on mitochondrial respiration.

References

1. Belhomme D, Peynet J, Louzy M, Launay JM, Kitakaze M, Menasche P: Evidence for preconditioning by isoflurane in coronary artery bypass graft surgery. *Circulation* 1999; 100:II340-4
2. Cason BA, Gamperl AK, Slocum RE, Hickey RF: Anesthetic-induced preconditioning: Previous administration of isoflurane decreases myocardial infarct size in rabbits. *ANESTHESIOLOGY* 1997; 87:1182-90
3. Coetzee A, Brits W, Genade S, Lochner A: Halothane does have protective properties in the isolated ischemic rat heart. *Anesth Analg* 1991; 73:711-9
4. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. *ANESTHESIOLOGY* 1997; 86:699-709
5. Davis RF, Sidi A: Effect of isoflurane on the extent of myocardial necrosis and on systemic hemodynamics, regional myocardial blood flow, and regional myocardial metabolism in dogs after coronary artery occlusion. *Anesth Analg* 1989; 69:575-86
6. Kanaya N, Fujita S: The effects of isoflurane on regional myocardial contractility and metabolism in "stunned" myocardium in acutely instrumented dogs. *Anesth Analg* 1994; 79:447-54
7. Kersten JR, Schmeling TJ, Hettrick DA, Pagel PS, Gross GJ, Wartler DC: Mechanism of myocardial protection by isoflurane. Role of adenosine triphosphate-regulated potassium (KATP) channels. *ANESTHESIOLOGY* 1996; 85:794-807
8. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Wartler DC: Isoflurane mimics ischemic preconditioning via activation of K(ATP) channels: Reduction of myocardial infarct size with an acute memory phase. *ANESTHESIOLOGY* 1997; 87:361-70
9. Mather S, Farhangkhgoee P, Karmazyn M: Cardioprotective effects of propofol and sevoflurane in ischemic and reperfused rat hearts: Role of K(ATP) channels and interaction with the sodium-hydrogen exchange inhibitor HOE 642 (cariporide). *ANESTHESIOLOGY* 1999; 91:1349-60
10. Oguchi T, Kashimoto S, Yamaguchi T, Nakamura T, Kumazawa T: Comparative effects of halothane, enflurane, isoflurane and sevoflurane on function and metabolism in the ischaemic rat heart. *Br J Anaesth* 1995; 74:569-75
11. Wartler DC, al-Wathiqi MH, Kampine JP, Schmeling WT: Recovery of contractile function of stunned myocardium in chronically instrumented dogs is enhanced by halothane or isoflurane. *ANESTHESIOLOGY* 1988; 69:552-65
12. Garlid KD, Pauczek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ: Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K⁺ channels. Possible mechanism of cardioprotection. *Circ Res* 1997; 81:1072-82
13. Liu Y, Sato T, O'Rourke B, Marban E: Mitochondrial ATP-dependent potassium channels: Novel effectors of cardioprotection? *Circulation* 1998; 97:2463-9
14. Sato T, O'Rourke B, Marban E: Modulation of mitochondrial ATP-dependent K⁺ channels by protein kinase C. *Circ Res* 1998; 83:110-4
15. Takashi E, Wang Y, Ashraf M: Activation of mitochondrial K(ATP) channel elicits late preconditioning against myocardial infarction via protein kinase C signaling pathway. *Circ Res* 1999; 85:1146-53
16. Yao Z, Gross GJ: Effects of the KATP channel opener bimakalim on coronary blood flow, monophasic action potential duration, and infarct size in dogs. *Circulation* 1994; 89:1769-75
17. Kohro S, Hogan QH, Nakae Y, Yamakage M, Bosnjak ZJ: Anesthetic effects on mitochondrial ATP-sensitive K channel. *ANESTHESIOLOGY* 2001; 95:1435-40
18. Chance B, Salkovitz IA, Kovach AG: Kinetics of mitochondrial flavoprotein and pyridine nucleotide in perfused heart. *Am J Physiol* 1972; 223:207-18
19. Xu ZL, Endoh H, Ishihata A, Takahashi E, Doi K: Effect of ischemic preconditioning on myocardial oxygen consumption during ischemia. *J Mol Cell Cardiol* 1998; 30:2165-74
20. Di Lisa F, Bernardi P: Mitochondrial function as a determinant of recovery or death in cell response to injury. *Mol Cell Biochem* 1998; 184:379-91
21. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM: Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res* 2000; 87:460-6