Anesthesiology 2003; 98:387–95

Preconditioning with Sevoflurane Reduces Changes in Nicotinamide Adenine Dinucleotide during Ischemia–Reperfusion in Isolated Hearts

Reversal by 5-Hydroxydecanoic Acid

Matthias L. Riess, M.D., Enis Novalija, M.D.,† Amadou K. S. Camara, Ph.D.,† Janis T. Eells, Ph.D.,‡ Qun Chen, M.D.,§ David F. Stowe, M.D., Ph.D.‖*

Background: **Ischemia causes an imbalance in mitochondrial metabolism and accumulation of nicotinamide adenine dinucleotide (NADH). We showed that anesthetic preconditioning (APC), like ischemic preconditioning, improved mitochondrial NADH energy balance during ischemia and improved function and reduced infarct size on reperfusion. Opening adenosine triphosphate–sensitive potassium (K_{ATP}) channels may be involved in triggering APC. The authors tested if effects of APC on NADH concentrations before, during, and after ischemia are reversible by 5-hydroxydecanoate (5-HD), a putative mitochondrial K_{ATP} channel blocker.**

Methods: **Nicotinamide adenine dinucleotide fluorescence was measured in 60 guinea pig Langendorff-prepared hearts assigned into five groups: (1) no treatment before ischemia; (2) APC by exposure to 1.3 mM sevoflurane for 15 min; (3) 200 μM 5-HD from 5 min before to 15 min after sevoflurane exposure; (4) 35 min 5-HD alone; and (5) no treatment and no ischemia. Sevoflurane was washed out for 30 min, and 5-HD for 15 min, before 30-min ischemia and 120-min reperfusion.**

Results: **Nicotinamide adenine dinucleotide was reversibly increased during sevoflurane exposure before ischemia, and the increase and rate of decline in NADH during ischemia were reduced after APC. 5-HD abolished these changes in NADH. On reperfusion, function was improved and infarct size reduced after APC compared with other groups.**

Conclusion: **Anesthetic preconditioning was evidenced by improved mitochondrial bioenergetics as assessed from NADH concentrations during ischemia and by attenuated reperfusion injury. Reversal of APC by bracketing sevoflurane exposure with 5-HD suggests that APC is triggered by mitochondrial K_{ATP} channel opening or, alternatively, by attenuated mitochondrial respiration without direct involvement of mitochondrial K_{ATP} channel opening.**

ANESTHETIC preconditioning (APC), *i.e.*, a temporary exposure to a volatile anesthetic followed by its complete washout, protects against cardiac ischemia–reperfusion (IR) injury. APC is as protective as ischemic preconditioning (IPC) in many models and has the distinct advantage that ischemia is not required to initiate preconditioning. APC reduced infarct size (%IS) in rabbits[1] and dogs.[2] Functionally, APC was manifested in guinea pig isolated hearts by less mechanical and metabolic dysfunction, reduced Ca^{2+} overload,[3] and improved coronary perfusion and drug-induced endothelial release of nitric oxide.[4] The exact triggering and cardioprotective mechanisms of APC, however, are not yet fully understood.

Opening of adenosine triphosphate–sensitive potassium (K_{ATP}) channels may be involved in triggering APC since several putative K_{ATP} channel antagonists attenuated cardioprotection induced by volatile anesthetics.[2,4–6] In support of this, diazoxide, a putative mitochondrial K_{ATP} (mK_{ATP}) channel opener, protected cardiac mitochondrial function during hypoxia[7] and induced cardioprotection.[8,9] The possible effect of mK_{ATP} channel opening on mitochondrial bioenergetics in the intact heart has not been examined.

One important marker of mitochondrial function is reduced nicotinamide adenine dinucleotide (NADH). Shortage of oxygen during ischemia leads to abnormal accumulation of NADH.[10,11] We have shown recently there is improved mitochondrial function during ischemia after APC, as evidenced by more normalized NADH fluorescence.[12] We hypothesized that the mitochondrial protection afforded by APC is mediated by mK_{ATP} channel opening. We examined if the putatively selective mK_{ATP} channel antagonist 5-hydroxydecanoate (5-HD) can reverse the changes in NADH and cardioprotective effects of APC before, during, and after ischemia in intact hearts.

Materials and Methods

Langendorff Heart Preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of

* Research Fellow, Anesthesiology Research Laboratories, Department of Anesthesiology, Medical College of Wisconsin, and Resident on leave, Westfälische-Wilhelms-Universität, Münster, Germany. † Instructor, § Research Fellow, Anesthesiology Research Laboratories, Department of Anesthesiology, ‡ Associate Professor, Department of Pharmacology and Toxicology, Medical College of Wisconsin. ‖ Professor, Anesthesiology Research Laboratories, Departments of Anesthesiology and Physiology, Cardiovascular Research Center, Medical College of Wisconsin and VA Medical Center Research Service, and Adjunct Professor, Department of Biomedical Engineering, Marquette University, Milwaukee, Wisconsin.

Received from the Anesthesiology Research Laboratories, Departments of Anesthesiology, Physiology, and Pharmacology and Toxicology, Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee Regional Medical Center, and the VA Medical Center Research Service, Milwaukee, Wisconsin. Submitted for publication May 7, 2002. Accepted for publication September 23, 2002. Supported in part by grants No. HL58691 (to Drs. Stowe and Camara) and ES06648 (to Dr. Eells) from the National Institutes of Health, Bethesda, Maryland, by grants No. 0020503Z (to Dr. Novalija) and 0151487Z (to Dr. Eells) from the American Heart Association, Dallas, Texas, and by grant No. Ri 610005 (to Dr. Riess) from the Medical Faculty of the Westfälische-Wilhelms-Universität, Münster, Germany. Dr. Riess received the First Prize in the 2002 American Society of Anesthesiologists Residents' Essay Contest for this study. Presented in part at the annual meetings of the International Anesthesia Research Society, San Diego, California, March 16–20, 2002, the European Society of Anaesthesiologists, Nice, France, April 6–9, 2002, and the American Society of Anesthesiologists, Orlando, Florida, October 12–16, 2002.

Address reprint requests to Dr. Stowe: M4250, 8701 Watertown Plank Road, Medical College of Wisconsin, Milwaukee, Wisconsin 53226. Address electronic mail to: dfstowe@mcw.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Health No. 85–23, revised 1996) and was approved by the institutional animal studies committee. Our methods have been described previously.[3,4,11,12] Thirty milligrams of ketamine and 1,000 units of heparin were injected intraperitoneally into 60 albino English short-haired guinea pigs (weight, 250–300 g). Ketamine given in this manner does not inhibit preconditioning, as reported when given before IPC in the isolated rat heart.[13] Animals were decapitated 15 min later when unresponsive to noxious stimulation. After thoracotomy, the aorta was cannulated distal to the aortic valve, and the heart was immediately perfused retrograde with 4°C cold oxygenated Krebs-Ringer solution. The inferior and superior venae cavae were ligated, and the heart was rapidly excised. After cannulation of the pulmonary artery to collect the coronary effluent, the heart was placed in the support system and perfused at 55 mmHg at 37°C. The Krebs-Ringer perfusate was equilibrated with ~97% O_2 and ~3% CO_2 to maintain a constant perfusion pH of 7.4 (pH, 7.40 ± 0.01; carbon dioxide partial pressure, 25 ± 2 mmHg; oxygen partial pressure, 570 ± 10 mmHg). The perfusate was filtered (5-μm pore size) in-line and had the following calculated composition (nonionized): 138 mM Na^+, 4.5 mM K^+, 1.2 mM Mg^{2+}, 2.5 mM Ca^{2+}, 134 mM Cl^-, 14.5 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 11.5 mM glucose, 2 mM pyruvate, 16 mM mannitol, 0.1 mM probenecid, 0.05 mM EDTA, and 5 U/l insulin.

Left ventricular pressure (LVP) was measured isovolumetrically with a saline-filled latex balloon inserted into the left ventricle through a cut in the left atrium. At the beginning of the experiment the balloon volume was adjusted to achieve a diastolic LVP of 0 mmHg, so that any subsequent increase in diastolic LVP reflected an increase in left ventricular wall stiffness, or diastolic contracture. Characteristic data from LVP were as follows: systolic, diastolic, systolic–diastolic LVP, and the maximal and minimal first derivatives of LVP ($dLVP/dt_{max}$ and $dLVP/dt_{min}$) as indices of contractility and relaxation, respectively. Spontaneous heart rate was monitored with bipolar electrodes placed in the right atrial and ventricular walls. Coronary flow (CF) was measured at constant temperature and perfusion pressure by an ultrasonic flowmeter (Transonic T106X, Ithaca, NY) placed directly into the aortic inflow line.

Coronary inflow (a) and coronary venous (v) Na^+, K^+, Ca^{2+}, oxygen partial pressure (Po_2), pH, and carbon dioxide partial pressure (Pco_2) were measured off-line with an intermittently self-calibrating analyzer system (Radiometer Copenhagen ABL 505, Copenhagen, Denmark). Po_2v tension was also measured continuously on-line with an O_2 Clark type electrode (model 203B; Instech, Plymouth Meeting, PA). Myocardial oxygen consumption (MVo_2) was calculated as $CF \cdot \text{heart wet weight}^{-1} \cdot (Po_2a - Po_2v) \cdot 24$ μl O_2/ml at 760 mmHg, and the cardiac efficiency index was calculated as $[sys - diaLVP] \cdot HR \cdot MVo_2^{-1}$.

Sevoflurane (Abbott Laboratories, Chicago, IL) was bubbled into the perfusate using an agent specific vaporizer (Vapor 2000; Dräger Medizintechnik GmbH, Lübeck, Germany) placed in the O_2–CO_2 gas mixture line. Samples of coronary perfusate were collected from a port in the aortic (inflow) and pulmonary cannulas (outflow) to measure sevoflurane concentrations by gas chromatography. Inflow and outflow sevoflurane concentrations were 1.3 ± 0.1 and 1.2 ± 0.1 mM, equivalent to 8.9 ± 0.7 and 8.3 ± 0.7% atmospheres at 37°C, respectively. These concentrations, which are too high to maintain general anesthesia but may be used temporarily during mask induction,[14,15] were chosen to unmask any possible effects on NADH concentrations during sevoflurane exposure or during IR.[12]

To potentially block mK_{ATP} channels, 5-HD (Sigma Chemical, St. Louis, MO) was given at a concentration of 200 μM.[8] If ventricular fibrillation occurred, a bolus of 250 μg lidocaine was immediately injected in the aortic cannula. All data were collected from hearts naturally in, or converted, to sinus rhythm. At the end of 120 min of reperfusion, hearts were removed and ventricles cut into six to seven transverse sections of 3-mm thickness. These were immediately stained with 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M KH_2PO_4 buffer (pH 7.4, 38°C) for 5 min. 2,3,5-Triphenyltetrazolium chloride stains viable tissue red, indicating the presence of a formazan precipitate that results from the reduction of 2,3,5-triphenyltetrazolium chloride by dehydrogenase enzymes present in viable tissue.[16] All slices were digitally imaged by a photoscanner, and the infarcted areas of each slice were measured in a blinded fashion by planimetry using software (Image 1.62) from the National Institutes of Health (Bethesda, MD). Infarcted areas of individual slices were averaged on the basis of their weight to calculate the total %IS of both ventricles (in percent). Reproducibility of this method is approximately ± 5% based on studies of fresh *ex vivo,* but nonperfused Langendorff prepared hearts (M. L. Riess, laboratory observations performed at Dr. David Stowe's laboratory at Medical College of Wisconsin).

Measurement of Mitochondrial Nicotinamide Adenine Dinucleotide in Intact Hearts

Tissue autofluorescence is a widely used and accepted method to measure NADH in isolated hearts and myocardial tissue.[10–12,17,18] To assess NADH fluorescence, each experiment was conducted in a light-blocking Faraday cage. The distal end of a bifurcated fiberoptic cable (6.8 mm^2 per bundle) was placed gently against the left ventricular anterior wall. A net was applied around the heart for optimal contact with the fiberoptic tip. This maneuver did not affect LVP. The two proximal ends of the fiberoptic cable were connected to a modified spectrophotometer (Photon Technology International, London, Canada). Fluorescence was excited with light from

a xenon arc lamp at 75 W. The light was filtered through a 350-nm monochromator (Delta RAM; Photon Technology International), and the beam was focused onto the in-going fibers of the optic bundle. The arc lamp shutter was opened only for 2.5-s recording intervals to prevent photobleaching; there were 26 recordings for a total exposure of 65 s over the course of each experiment. Fluorescence emissions were collected by fibers of the second limb of the cable. This light was separated by a dichroic beamsplitter at 430 nm and filtered by interference filters (Chroma Technology Corp., Brattleboro, VT) at 405 ± 15 and 460 ± 10 nm. Intensities were measured by photomultipliers (Photomultiplier Detection System 814; Photon Technology International, London, Canada).

Although autofluorescence at 460 nm could also arise from unknown intracellular constituents or cytosolic NADH, the majority is derived from mitochondrial NADH.[17,19] Motion artifacts in the NADH fluorescence at 460 nm are diminished by using 405 nm as a second reference wavelength that is less sensitive to changes in NADH; thus, the ratio of the intensities at 460 and at 405 nm is interpreted as a measure of NADH.[18] The use of these two tissue light isobestic wavelengths also accounts for possible alterations in myoglobin light absorption, *e.g.*, by hypoxia.[18] Since we did not calibrate NADH fluorescence, we obtained more conservative estimates of changes in NADH.[20] NADH is given in arbitrary fluorescence units.

All analog signals were digitized (PowerLab/16 SP; ADInstruments, Castle Hills, Australia) and recorded at 200 Hz (Chart & Scope v3.6.3; ADInstruments) on Power Macintosh® Computer G4 (Apple, Cupertino, CA) for later analysis using MATLAB® (The MathWorks, Natick, MA) and Microsoft Excel® (Microsoft Corporation, Redmond, WA) software. All variables were averaged over the sampling period of 2.5 s.

Protocol

After stabilization, each experiment lasted 200 min (fig. 1). Hearts were randomly assigned to one of five groups: (1) Untreated ischemic control hearts (ISC, n = 12) were not subjected to preconditioning or given the drug 5-HD. (2) Preconditioned hearts (APC, n = 12) were exposed to sevoflurane for 15 min, followed by a 30-min washout period before ischemia. (3) Other hearts were exposed to sevoflurane and 5-HD (APC+5-HD, n = 12); in this group sevoflurane exposure was bracketed by perfusion with 200 μM 5-HD from 5 min before to 15 min after sevoflurane exposure, followed by 15-min washout before ischemia. (4) Additional hearts were exposed to 5-HD only (5-HD, n = 12) for 35 min, followed by 15-min washout before ischemia. (5) Nonischemia time control hearts (CON) were perfused for 200 min. There were no differences in sevoflurane concentrations between APC and APC+5-HD hearts. Sevoflurane was essentially undetectable (0.05 ± 0.01 mM) in the effluent 30 min after its washout before ischemia. All ischemia hearts then underwent 30 min of global ischemia and 120 min of reperfusion.

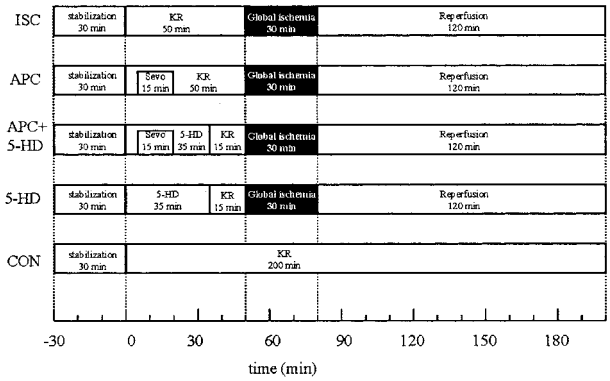

Fig. 1. Protocol for five randomized groups of guinea pig hearts (n = 12 in each group). After initial stabilization, anesthetic preconditioning (APC) was elicited by exposing hearts to 1.3 mM sevoflurane for 15 min. 5-Hydroxydecanoate (5-HD; 200 μM) was perfused alone or before, during, and after sevoflurane administration for 35 min. Sevoflurane was washed out for 30 min and 5-HD for 15 min. All ischemic hearts then underwent 30 min of global no-flow ischemia and 120 min of reperfusion; nonischemic hearts (CON) served as controls over time.

Statistical Analysis

All data are expressed as mean ± SEM. For among-group data, ISC, APC, APC+5-HD, 5-HD, and CON were compared by analysis of variance to determine significance (Super ANOVA 1.11® software for Macintosh®; Abacus Concepts, Berkeley, CA) at the following selected time points: before (at 0 and 5 min), during (at 20 min), and after (at 35 and 50 min) sevoflurane exposure; during ischemia (at 55 and 80 min); and during reperfusion (at 85, 140, and 200 min). If F values ($P < 0.05$) were significant, *post hoc* comparisons of means tests (Student-Newman-Keuls) were used to compare the five groups. Differences among means were considered statistically significant when $P < 0.05$ (two-tailed). Statistical symbols used were: [a]ISC, [b]APC, [c]APC+5-HD, and [d]5-HD *versus* CON; [e]APC, [f]APC+5-HD, and [g]5-HD *versus* ISC; [h]APC+5-HD and [i]5-HD *versus* APC; and [j]5-HD *versus* APC+5HD. Regression–correlation analyses were used to determine relations between (1) %IS and the rate of the NADH decline during ischemia ($dNADH_I/dt$), and (2) %IS and the NADH deviation from baseline ($\Delta NADH_{RP}$) at 120 min of reperfusion.

Results

Changes in Nicotinamide Adenine Dinucleotide Fluorescence

Figure 2 shows the changes in NADH for each of the four ischemia groups before, during, and after ischemia and for the nonischemia time control group. NADH remained stable for 140 min in the nonischemia CON

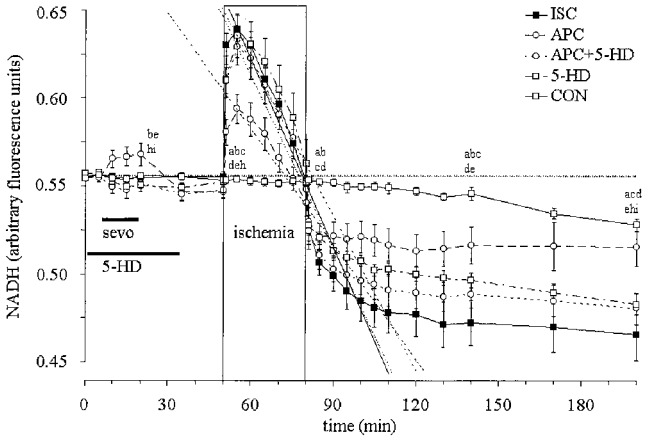

Fig. 2. Time course of nicotinamide adenine dinucleotide (NADH) in arbitrary fluorescence units for each of the five groups. Sevoflurane exposure caused a reversible increase in NADH that was blocked by 5-hydroxydecanoate (5-HD). At the onset of ischemia, NADH increased in all ischemic groups; this was followed by a gradual decline toward preischemic concentrations. Anesthetic preconditioning (APC) hearts showed a lower initial increase ($P < 0.05$) and a slower decline (as indicated by the dotted slopes) during prolonged ischemia than did untreated ischemic control hearts (ISC), APC+5-HD, and 5-HD hearts ($P < 0.05$). Throughout reperfusion, NADH fluorescence deviated the least from preischemic concentrations in APC hearts and was not different from nonischemia time control hearts (CON) at the 120 min of reperfusion. Statistical symbols for $P < 0.05$: [a]ISC, [b]APC, [c]APC+5-HD, and [d]5-HD *versus* CON; [e]APC, [f]APC+5-HD, and [g]5-HD *versus* ISC; [h]APC+5-HD and [i]5-HD *versus* APC; and [j]5-HD *versus* APC+5HD.

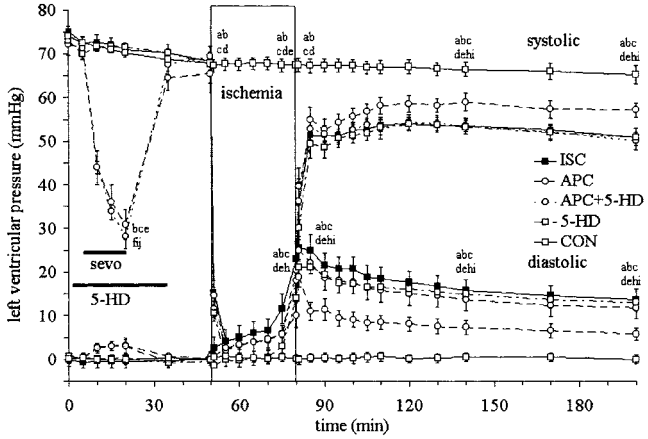

Fig. 3. Time course of left ventricular pressure (LVP) for each of the five groups. Sevoflurane exposure caused a completely reversible decrease in systolic LVP. 5-Hydroxydecanoate (5-HD) before, during, and after sevoflurane exposure did not alter this decrease; 5-HD alone did not change LVP significantly. Diastolic LVP increased similarly in each ischemic group during the final 5 min of ischemia. After 30 min of reperfusion, anesthetic preconditioning (APC) hearts exhibited higher systolic LVP and a lower elevation of diastolic LVP compared with untreated ischemic control hearts (ISC), APC+5-HD, and 5-HD hearts. See figure 2 for statistical symbols.

group and then slowly decreased by about 5% at 200 min. Exposure to 1.3 mM sevoflurane caused a reversible increase in NADH. This was fully blocked when bracketed by 5-HD. For all ischemia groups, NADH increased during initial ischemia; this was followed by a gradual decline toward preischemic concentrations. APC hearts showed a significantly lower initial increase in NADH and a slower decline ($P < 0.05$) during prolonged ischemia than did CON hearts. Bracketing 5-HD during sevoflurane exposure before ischemia abolished these differences during ischemia. Throughout reperfusion, NADH fluorescence deviated the least from preischemic concentrations in the APC group and was not different from the CON group after 120 min of reperfusion. 5-HD given before ischemia had a similar effect on NADH fluorescence before, during, and after ischemia as the CON group.

Changes in Mechanical Function

Figure 3 shows changes in LVP for each of the four ischemia groups before, during, and after ischemia and for the nonischemia time control group. Systolic LVP decreased slightly over the 200-min time course of the experiment, as evidenced in the CON group; diastolic LVP remained constant. Sevoflurane exposure caused a large but completely reversible decrease in systolic LVP. 5-HD before, during, and after sevoflurane exposure did not alter this decrease; 5-HD alone did not alter systolic LVP. Diastolic LVP increased in each ischemic group during the final 5–10 min of ischemia and was lower at 30 min of ischemia in the APC group than in the ISC and APC+5-HD groups. Throughout reperfusion, diastolic LVP was lower in the APC group than in the ISC, APC+5-HD, and 5-HD groups. From 60 min of reperfusion on, systolic LVP was higher in APC hearts compared with ISC, APC+5-HD, and 5-HD hearts. Developed (systolic − diastolic) LVP (data not displayed) was also improved throughout reperfusion in the APC group compared with all other ischemic groups ($P < 0.05$).

Figure 4 shows changes in $dLVP/dt_{max}$ (contractility) and $dLVP/dt_{min}$ (relaxation) for each of the four ischemia groups before, during, and after ischemia and for the CON group. $dLVP/dt_{max}$ and $dLVP/dt_{min}$ remained stable in CON hearts over the 200-min experiment. $dLVP/dt_{max}$ and $dLVP/dt_{min}$ were reversibly depressed during sevoflurane exposure. 5-HD before, during, and after sevoflurane exposure did not alter this decrease; 5-HD alone did not alter $dLVP/dt_{max}$ and $dLVP/dt_{min}$. During reperfusion, the contraction and relaxation indices were improved only in APC hearts and were not different among the ISC, APC+5-HD, and 5-HD hearts.

Sevoflurane exposure altered the heart rate reversibly from 247 ± 5 beats/min in CON hearts and 241 ± 7 beats/min in ISC hearts to 173 ± 6 in APC and 182 ± 15 beats/min in APC+5-HD hearts, respectively ($^{bcefij}P < 0.05$). Heart rate recovered fully on washout of sevoflurane before ischemia. 5-HD alone did not affect the heart rate (250 ± 7 beats/min). At all other perfusion periods, there was no significant difference among the groups (data not displayed). Ventricular fibrillation occurred in

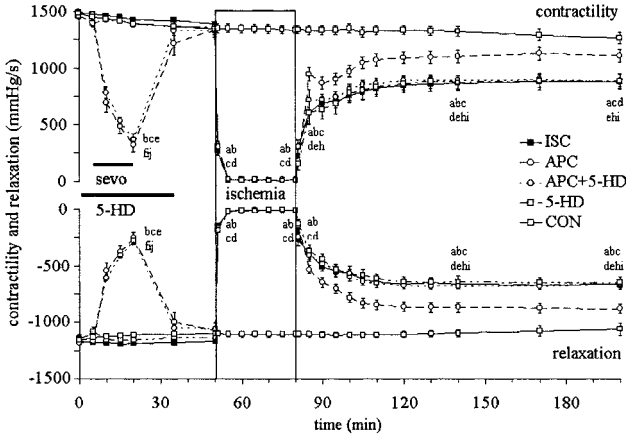

Fig. 4. Time course of $dLVP/dt_{max}$ (contractility) and $dLVP/dt_{min}$ (relaxation) for each of the five groups. $dLVP/dt_{max}$ and $dLVP/dt_{min}$ were reversibly depressed during sevoflurane exposure; this was not blocked by 5-hydroxydecanoate (5-HD) bracketing. During reperfusion, the contraction and relaxation indices were improved only in anesthetic preconditioning (APC) hearts compared with untreated ischemic control hearts (ISC), APC+5-HD, and 5-HD hearts. See figure 2 for statistical symbols.

each ischemia group. In each incident, ventricular fibrillation was converted immediately to sinus rhythm with lidocaine. There were no differences in the incidence of ventricular fibrillation among the ischemia groups.

Changes in Metabolic Function

Figure 5 shows changes in coronary flow for each of the five groups. Coronary flow increased slightly over the 200-min experiment as evidenced in the CON group. Coronary flow reversibly increased during sevoflurane exposure. 5-HD before, during, and after sevoflurane

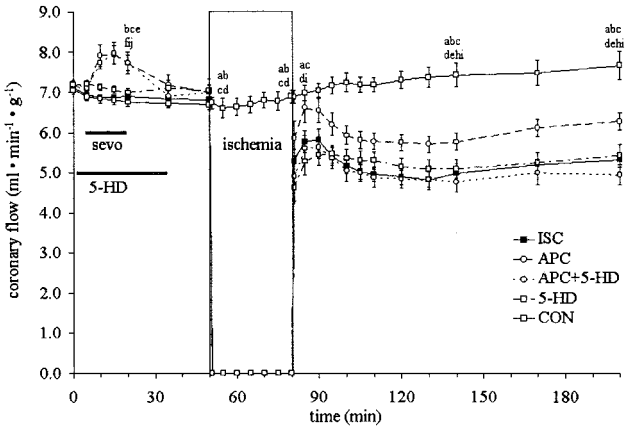

Fig. 5. Time course of coronary flow (CF) for each of the five groups. CF increased slightly during the 200-min time course in the nonischemia time control (CON) group. CF reversibly increased during sevoflurane exposure. CF was not altered by 5-hydroxydecanoate (5-HD) before, during, and after sevoflurane exposure. 5-HD alone did not effect CF. All ischemia groups exhibited a similar postischemic flow response on early reperfusion. During later reperfusion, coronary flow recovered better in the anesthetic preconditioning (APC) group than in other ischemia groups but did not reach CON values by the end of the experiment. See figure 2 for statistical symbols.

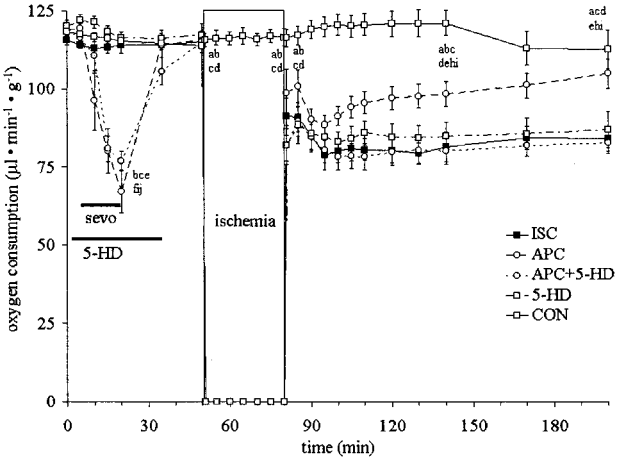

Fig. 6. Time course of mitochondrial oxygen consumption (Mvo_2) for each of the five groups. Mvo_2 was reversibly decreased during sevoflurane exposure. 5-Hydroxydecanoate (5-HD) before, during, and after sevoflurane exposure did not alter this decrease. 5-HD alone had no significant effect on Mvo_2. During reperfusion, Mvo_2 steadily improved in anesthetic preconditioning (APC) hearts and reached nonischemia time control (CON) values by the end of the experiment. In all other ischemia groups, Mvo_2 remained lower. See figure 2 for statistical symbols.

exposure did not alter this increase; 5-HD alone did not affect coronary flow. All ischemia groups exhibited a similar postischemic flow response on early reperfusion. During later reperfusion, coronary flow recovered better in the APC group than in other ischemia groups but did not reach CON values by the end of the experiment.

Myocardial oxygen consumption is displayed in figure 6. It was also reversibly decreased during sevoflurane exposure; 5-HD before, during, and after sevoflurane exposure did not alter this decrease. 5-HD alone had no effect on Mvo_2. During reperfusion, Mvo_2 steadily improved in APC hearts and reached CON values by the end of the experiment. In all other ischemia groups, Mvo_2 remained lower.

The cardiac efficiency index (heart rate · [systolic − diastolic LVP] · Mvo_2^{-1}) (fig. 7) was also reversibly decreased during sevoflurane exposure. 5-HD before, during, and after sevoflurane exposure did not alter this decrease. 5-HD alone had no significant effect on this index. The cardiac efficiency index steadily improved in all ischemic groups during the first 30 min of reperfusion but remained below CON values. The index was not different in CON and APC groups by the end of the experiment.

Infarct Size and Its Prediction during Ischemia and Reperfusion

Figure 8 shows that APC resulted in decreased cardiac %IS. This decrease was abolished by bracketing sevoflurane exposure with 5-HD before ischemia. 5-HD alone had no effect on %IS. As shown in figure 9A, there was a significant correlation between %IS and the rate of

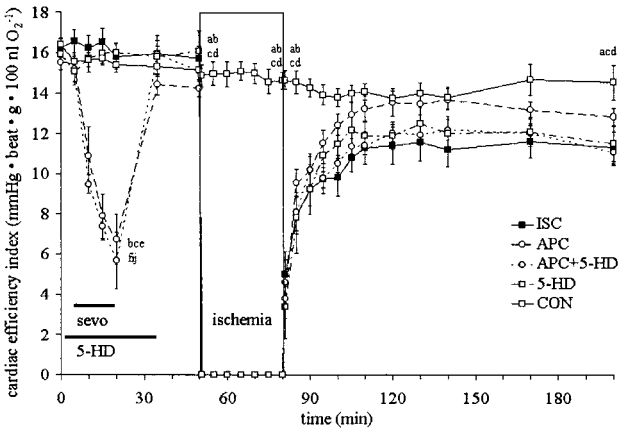

Fig. 7. Time course of the cardiac efficiency index (heart rate · [systolic − diastolic left ventricular pressure]/mitochondrial oxygen consumption) for each of the five groups. The index was reversibly decreased during sevoflurane exposure. 5-Hydroxydecanoate (5-HD) before, during, and after sevoflurane exposure did not alter this decrease. 5-HD alone had no significant effect on the cardiac efficiency index. The index steadily improved in all ischemia groups during the first 30 min of reperfusion but remained below nonischemia time control (CON) values. Only anesthetic preconditioning (APC) hearts were not different from CON hearts by the end of the experiment. See figure 2 for statistical symbols.

NADH decline ($-dNADH_I/dt$ in arbitrary fluorescence units per minute) from the peak increase in NADH at 5 min ischemia to the end of ischemia in the 48 ischemic hearts. We also found a significant correlation between %IS and the deviation of NADH ($\Delta NADH_{RP}$ in arbitrary fluorescence units) from baseline values at 120 min of reperfusion in all 60 hearts (fig. 9B). Both of these data sets were better fit by a linear than by a nonlinear relation (form of y = a + bx). See details in figure legend.

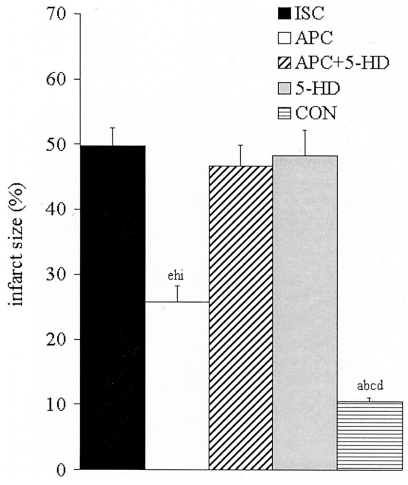

Fig. 8. Infarct size was reduced by anesthetic preconditioning (APC); this reduction was abolished by bracketing sevoflurane exposure with 5-hydroxydecanoate (5-HD) before ischemia. 5-HD alone had no effect on infarct size. All ischemia groups were different for infarct size compared with the nonischemia time control group. See figure 2 for statistical symbols.

Discussion

Anesthetic preconditioning is well known to attenuate cardiac IR injury, but its underlying mechanisms have not been fully elucidated. In particular, it is unknown how anesthetics trigger preconditioning. This study demonstrates that administration of 5-HD, presumed to be a selective mK_{ATP} channel blocker, not only prevented functional, metabolic, and tissue cardioprotection by APC on reperfusion in guinea pig isolated hearts, but also reversed APC-induced changes in mitochondrial NADH before and during IR. The capability to assess changes in NADH continuously as a marker of mitochondrial bioenergetics in the intact heart furnishes additional insight into the mechanism of APC-induced cardioprotection.

Anesthetic Preconditioning: Putative Role of Adenosine Triphosphate–Sensitive Potassium Channels

Adenosine triphosphate–sensitive potassium channel opening is thought to be an important mechanism in APC as well as IPC. This is based on observations that several putative sarcolemmal K_{ATP} and mK_{ATP} channel antagonists attenuate preconditioning and cardioprotection induced by volatile anesthetics.[2,4–6] Because 5-HD abrogated the changes in NADH induced by APC, our results suggest in addition that mK_{ATP} channel opening is involved in providing the protective mitochondrial effect. Additional evidence for involvement of the mK_{ATP} channel is provided by the putative mK_{ATP} channel opener diazoxide, which appears to mimic IPC on %IS[8] and to afford protection during ischemia.[21–23] However, there is a debate over the role of sarcolemmal K_{ATP} channels and if mK_{ATP} channels have a greater role in triggering or effecting preconditioning.[9,24]

Although opening mK_{ATP} channels may be an effective way to attenuate mitochondrial damage, it remains unclear how mK_{ATP} channel opening might protect mitochondrial function. mK_{ATP} channel opening and subsequent mitochondrial K^+ uptake has been reported to cause a significant matrix swelling and a small decrease in the negative mitochondrial membrane potential.[25] Other data suggest that K^+ influx partially dissipates the mitochondrial membrane potential and, if compensated by an acceleration of electron transfer, leads to a net oxidation in the mitochondria. This was demonstrated by an increase in flavoprotein fluorescence by diazoxide in rabbit ventricular myocytes.[26]

Mitochondrial ATP synthesis at Complex V (F_1F_o-ATP synthetic complex) is driven by the electrochemical proton gradient in the inner mitochondrial membrane that is generated by the transfer of electrons from NADH (and $FADH_2$) to oxygen.[27] Complex I (NADH CoQ oxidoreductase) is the first stage of proton and electron donation for NADH. Complex III (ubiquinol:ferricyto-

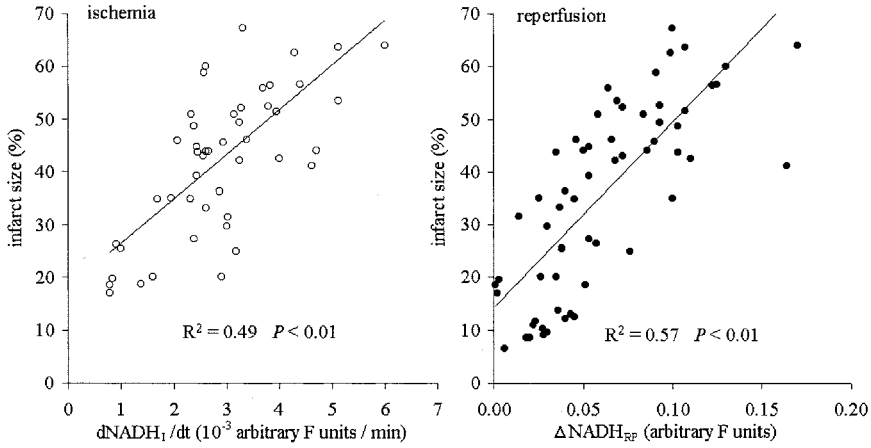

Fig. 9. (*A*) Correlation between infarct size (%IS) and the rate of the nicotinamide adenine dinucleotide (NADH) decline ($-dNADH_I/dt$ in arbitrary fluorescence units per minute) from the peak increase in NADH at 5 min of ischemia to the end of ischemia. The relation was $\%IS = 18 \pm 4 + (8.4 \pm 1.2) \cdot 10^3\ dNADH_I/dt$; $R^2 = 0.49$; $P < 0.01$; n = 48. (*B*) Correlation between %IS and the deviation of NADH ($\Delta NADH_{RP}$ in arbitrary fluorescence units) from baseline values at 120 min of reperfusion. The relation was $\%IS = 14 \pm 3 + 355 \pm 41 \cdot \Delta NADH_{RP}$; $R^2 = 0.57$; $P < 0.01$; n = 60.

chrome c oxidoreductase) spans the central part of the respiratory chain, catalyzing the electron transfer from ubiquinol to oxidized cytochrome c, which is also coupled to proton translocation. With adequate substrate and oxygen, this system is in equilibrium, and oxidation of NADH to NAD^+ and phosphorylation of adenosine diphosphate to ATP are matched. NADH and $FADH_2$ are products of the tricarboxylic acid cycle and the β-oxidation of fatty acids.[28] Dehydrogenases generate NADH from NAD^+ during glycolysis. Steady state NADH concentrations are therefore determined by the balance between NADH generation and oxidation. During ischemia, *e.g.*, the supply of oxygen to accept electrons diminishes, electron flux through the electron transport chain falters, and NADH accumulates.[11,12,29]

Mitochondrial Energetics during Sevoflurane Exposure

We reported recently[12] that either a temporary exposure to a high concentration of sevoflurane or brief ischemia (IPC) reversibly increased NADH in normothermic guinea pig intact hearts but offered no mechanism. The current study offers the first evidence that 5-HD given before, during, and after anesthetic exposure not only prevents the preconditioning stimulus and subsequent cardioprotective effects, but also completely abolishes the anesthetic-induced increase in NADH. If 5-HD is purely a mK_{ATP} channel blocker, this would suggest that direct or indirect opening of mK_{ATP} channels is involved in the triggering mechanism of APC and that the anesthetic-induced increase in NADH is a consequence of the mK_{ATP} channel opening.

Another possibility is that volatile anesthetics directly inhibit complex I[30,31] to cause an increase in NADH. A concentration-dependent increase in NADH fluorescence was reported earlier with exposure to other volatile anesthetics in an isolated rat heart model.[32] Increased NADH caused by complex I inhibition may cause the formation of reactive oxygen species. Reactive oxygen species in turn have been shown to activate K_{ATP} channels.[33,34] This could create a feed-forward interaction between NADH accumulation (decreased oxidation) and K_{ATP} channel opening that might be inhibited by blocking the mK_{ATP} channel.

Direct blockade of complex I activity by volatile anesthetics would also be expected to lead to a compensatory increase in electron transfer *via* flavoprotein oxidation at complex II. Indeed, a recent study[35] showed that a comparable sevoflurane concentration mildly increased flavoprotein fluorescence (increased oxidation) in isolated myocytes bathed in a substrate-free solution. This would imply then that 5-HD, like diazoxide,[36] may not exclusively target the mK_{ATP} channel. Indeed, 5-HD may functionally antagonize the inhibition of the respiratory chain by volatile anesthetics at complex I and diazoxide at complex II, as recently suggested by Hanley *et al.*[37] 5-HD, as a substrate for acyl-CoA synthetase, could provide a bypass for electrons around complex I or II to ubiquinone at complex III. Their study raises a serious concern about the assumed selectivity of diazoxide and 5-HD for the mK_{ATP} channel. Our data are also compatible with their theory because 5-HD reversed the sevoflurane-induced increase in NADH. Therefore, any definite conclusions on the role of the mK_{ATP} channel using these drugs must be limited at this point. Clearly, a rigorous examination of the coupling of electron transport chain and oxidative phosphorylation during anesthetic exposure will be required from experiments in isolated mitochondria and isolated myocytes, as well as in intact hearts, to more specifically address the triggering mechanisms of APC.

Mitochondrial Energetics during Ischemia–Reperfusion after Sevoflurane Exposure

As we described previously, a sudden shortage in cellular oxygen during the onset of ischemia leads to a rapid imbalance between NADH generation and oxidation.[12] In the current study, NADH increased and peaked in all groups at 5 min of ischemia. The decline in NADH after 5 min of ischemia may be interpreted as a relatively lower rate of NADH generation than oxidation during prolonged ischemia. APC hearts showed a slower NADH

decline; this could reflect a slower remaining rate of oxidative phosphorylation or a higher dehydrogenase activity or larger substrate stores compared with control hearts. Giving 5-HD to bracket exposure to the anesthetic abolished both the lower initial NADH increase as well as the slower NADH decline during prolonged ischemia. This suggests that the lesser changes in NADH during ischemia after sevoflurane exposure are caused by the preconditioning effect of sevoflurane, rather than being merely accompanied by the previous exposure to sevoflurane *per se*. Similarly, because the same degree of temporary and fully reversible cardiac depression (contractility, coronary flow, Mvo_2, cardiac efficiency) during sevoflurane exposure was observed in APC and APC+5-HD hearts, this temporary cardiac depression alone cannot be responsible for the subsequent APC. Temporary cardiac depression with other drugs such as pentobarbital or propofol does not necessarily lead to preconditioning.[38]

The overall NADH fluorescence signal measured in this study is a product of average NADH per cell and the number of viable cells contributing to the observed fluorescence. Infarction in our model is concentric and subepicardial. Thus, infarcted cells as well as viable cells underlie the fiberoptic probe on reperfusion. Because there was either incomplete or no recovery in NADH fluorescence over time on reperfusion, and because the magnitude of the deviation correlated with %IS, the observed decrease in tissue fluorescence could be a result of infarcted tissue not contributing to the overall signal rather than to a permanent decrease in NADH per cell. In contrast to the reduced NADH fluorescence in ISC, APC+5-HD, and 5-HD hearts, its relative normalization in APC hearts may be evidence of greater cell salvage.

Limitations and Summary

We used an isolated heart model with a blood-free perfusate. *In vivo*, volatile anesthetics may modify neutrophil function related to IR injury. IR injury may also be modified by hormonal input or the autonomic nervous system. Many investigators have used other species, particularly the rat, as a model for APC, but the guinea pig heart is believed to be closer in design to the human heart.[3,39–41] We used only one pulse of high concentration sevoflurane to induce APC in this study, but we have shown previously that sevoflurane has a concentration-dependent effect to induce APC[12] and that APC can be induced with two pulses at lower concentrations of sevoflurane.[3,4] Finally, it is important to acknowledge that any conclusion on the role of mK_{ATP} channel opening depends on the selectivity of 5-HD for blocking the mK_{ATP} channel.

In conclusion, 5-HD, a putative mitochondrial K_{ATP} channel blocker, not only abolished protection induced by APC against mechanical dysfunction and tissue necrosis on reperfusion, but also reversed all changes in mitochondrial energetics induced by APC before, during, and after ischemia, as evidenced by the changes in NADH fluorescence. Our study also demonstrates that subsequent %IS can be predicted by changes in NADH during ischemia and by the deviation of NADH fluorescence during reperfusion. The reversible increase of NADH by sevoflurane exposure before ischemia suggests a blockade of mitochondrial electron transport is part of the triggering mechanism of APC and that its reversal by 5-HD is consistent with an mK_{ATP}-independent action of 5-HD.[37] If we assume that the results of our research are applicable to patients at risk for cardiac ischemia during surgery,[42] then even brief exposure to a volatile anesthetic may be beneficial in the perioperative period.

The authors thank Ming Tao Jiang, Ph.D., James Heisner, B.S., and Samhita Rhodes, M.S. (all Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin), for their valuable contributions to this study.

References

1. 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
2. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier 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
3. An JZ, Varadarajan SG, Novalija E, Stowe DF: Ischemic and anesthetic preconditioning reduces cytosolic $[Ca^{2+}]$ and improves Ca^{2+} responses in intact hearts. Am J Physiol Heart Circ Physiol 2001; 281:H1508–23
4. Novalija E, Fujita S, Kampine JP, Stowe DF: Sevoflurane mimics ischemic preconditioning effects on coronary flow and nitric oxide release in isolated hearts. ANESTHESIOLOGY 1999; 91:701–12
5. Toller WG, Gross ER, Kersten JR, Pagel PS, Gross GJ, Warltier DC: Sarcolemmal and mitochondrial adenosine triphosphate-dependent potassium channels: Mechanism of desflurane-induced cardioprotection. ANESTHESIOLOGY 2000; 92: 1731–9
6. Piriou V, Chiari P, Knezynski S, Bastien O, Loufoua J, Lehot JJ, Foex P, Annat G, Ovize M: Prevention of isoflurane-induced preconditioning by 5-hydroxydecanoate and gadolinium: Possible involvement of mitochondrial adenosine triphosphate-sensitive potassium and stretch-activated channels. ANESTHESIOLOGY 2000; 93:756–64
7. Ozcan C, Holmuhamedov EL, Jahangir A, Terzic A: Diazoxide protects mitochondria from anoxic injury: Implications for myopreservation. J Thorac Cardiovasc Surg 2001; 121:298–306
8. Garlid KD, Paucek 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
9. Gross GJ: The role of mitochondrial K_{ATP} channels in cardioprotection. Basic Res Cardiol 2000; 95:280–4
10. Chance B, Williamson JR, Jamieson D, Schoenner B: Properties and kinetics of reduced pyridine nucleotide fluorescence of the isolated and in vivo rat heart. Biochem Z 1965; 341:357–77
11. Varadarajan SG, An JZ, Novalija E, Smart SC, Stowe DF: Changes in $[Na^+]_i$, compartmental $[Ca^{2+}]$, and NADH with dysfunction after global ischemia in intact hearts. Am J Physiol Heart Circ Physiol 2001; 280:H280–93
12. Riess ML, Camara AK, Chen Q, Novalija E, Rhodes SS, Stowe DF: Altered NADH and improved function by anesthetic and ischemic preconditioning in guinea pig intact hearts. Am J Physiol Heart Circ Physiol 2002; 283:H53–60
13. Molojavyi A, Preckel B, Comfere T, Mullenheim J, Thamer V, Schlack W: Effects of ketamine and its isomers on ischemic preconditioning in the isolated rat heart. ANESTHESIOLOGY 2001; 94:623–9
14. Epstein RH, Stein AL, Marr AT, Lessin JB: High concentration versus incremental induction of anesthesia with sevoflurane in children: A comparison of induction times, vital signs, and complications. J Clin Anesth 1998; 10:41–5
15. Walpole R, Logan M: Effect of sevoflurane concentration on inhalation induction of anaesthesia in the elderly. Br J Anaesth 1999; 82:20–4
16. Altman FP: Tetrazolium salts and formazans. Prog Histochem Cytochem 1976; 9:1–56

17. Nuutinen EM: Subcellular origin of the surface fluorescence of reduced nicotinamide nucleotides in the isolated perfused rat heart. Basic Res Cardiol 1984; 79:49–58

18. Brandes R, Bers DM: Increased work in cardiac trabeculae causes decreased mitochondrial NADH fluorescence followed by slow recovery. Biophys J 1996; 71:1024–35

19. Eng J, Lynch RM, Balaban RS: Nicotinamide adenine dinucleotide fluorescence spectroscopy and imaging of isolated cardiac myocytes. Biophys J 1989; 55:621–30

20. Brandes R, Bers DM: Analysis of the mechanisms of mitochondrial NADH regulation in cardiac trabeculae. Biophys J 1999; 77:1666–82

21. Xu M, Wang Y, Ayub A, Ashraf M: Mitochondrial K_{ATP} channel activation reduces anoxic injury by restoring mitochondrial membrane potential. Am J Physiol Heart Circ Physiol 2001; 281:H1295–303

22. Wang S, Cone J, Liu Y: Dual roles of mitochondrial K_{ATP} channels in diazoxide-mediated protection in isolated rabbit hearts. Am J Physiol Heart Circ Physiol 2001; 280:H246–55

23. Fryer R, Hsu AK, Gross GJ: Mitochondrial K_{ATP} channel opening is important during index ischemia and following myocardial reperfusion in ischemic preconditioned rat hearts. J Mol Cell Cardiol 2001; 33:831–4

24. O'Rourke B: Myocardial K_{ATP} channels in preconditioning. Circ Res 2000; 87:845–55

25. Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD: Bioenergetic consequences of opening the ATP-sensitive K^+ channel of heart mitochondria. Am J Physiol Heart Circ Physiol 2001; 280:H649–57

26. Liu Y, Sato T, O'Rourke B, Marban E: Mitochondrial ATP-dependent potassium channels: Novel effectors of cardioprotection? Circulation 1998; 97: 2463–9

27. Cairns CB, Ferroggiaro AA, Walther JM, Harken AH, Banerjee A: Postischemic administration of succinate reverses the impairment of oxidative phosphorylation after cardiac ischemia and reperfusion injury. Circulation 1997; 96:II-260–5

28. Scholz TD, Laughlin MR, Balaban RS, Kupriyanov VV, Heineman FW: Effect of substrate on mitochondrial NADH, cytosolic redox state, and phosphorylated compounds in isolated hearts. Am J Physiol Heart Circ Physiol 1995; 268:H82–91

29. Ferrari R, Cargnoni A, Bernocchi P, Pasini E, Curello S, Ceconi C, Ruigrok TJ: Metabolic adaptation during a sequence of no-flow and low-flow ischemia: A possible trigger for hibernation. Circulation 1996; 94:2587–96

30. Nahrwold ML, Cohen PJ: Anesthetics and mitochondrial respiration. Clin Anesth 1975; 11:25–44

31. Cohen PJ: Effect of anesthetics on mitochondrial function. ANESTHESIOLOGY 1973; 39:153–64

32. Kissin I, Aultman DF, Smith LR: Effects of volatile anesthetics on myocardial oxidation-reduction status assessed by NADH fluorometry. ANESTHESIOLOGY 1983; 59:447–52

33. Tokube K, Kiyosue T, Arita M: Openings of cardiac K_{ATP} channel by oxygen free radicals produced by xanthine oxidase reaction. Am J Physiol Heart Circ Physiol 1996; 271:H478–89

34. Tokube K, Kiyosue T, Arita M: Effects of hydroxyl radicals on K_{ATP} channels in guinea-pig ventricular myocytes. Eur J Physiol 1998; 437:155–7

35. Kohro S, Hogan QH, Nakae Y, Yamakage M, Bosnjak ZJ: Anesthetic effects on mitochondrial ATP-sensitive K channel. ANESTHESIOLOGY 2001; 95:1435–440

36. Grimmsmann T, Rustenbeck I: Direct effects of diazoxide on mitochondria in pancreatic B-cells and on isolated liver mitochondria. Br J Pharmacol 1998; 123:781–8

37. Hanley PJ, Mickel M, Loffler M, Brandt U, Daut J: K_{ATP} channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. J Physiol 2002; 542:735–41

38. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. ANESTHESIOLOGY 1997; 86:699–709

39. Steinfath M, Chen YY, Lavicky J, Magnussen O, Nose M, Rosswag S, Schmitz W, Scholz H: Cardiac alpha 1-adrenoceptor densities in different mammalian species. Br J Pharmacol 1992; 107:185–8

40. Kanai A, Salama G: Optical mapping reveals that repolarization spreads anisotropically and is guided by fiber orientation in guinea pig hearts. Circ Res 1995; 77:784–802

41. Takeishi Y, Huang Q, Wang T, Glassman M, Yoshizumi M, Baines CP, Lee JD, Kawakatsu H, Che W, Lerner-Marmarosh N, Zhang C, Yan C, Ohta S, Walsh RA, Berk BC, Abe J: Src family kinase and adenosine differentially regulate multiple MAP kinases in ischemic myocardium: Modulation of MAP kinases activation by ischemic preconditioning. J Mol Cell Cardiol 2001; 33:1989–2005

42. 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