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Nitric oxide has no obligatory role in isoflurane late preconditioning against myocardial stunning

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ABSTRACT

Aims: Isoflurane has been demonstrated to produce late preconditioning against myocardial stunning. We tested the hypothesis that this effect is dependent upon an increased production of nitric oxide.
Main Methods: Studies were performed in 18 conscious dogs, chronically instrumented to measure coronary blood flow and myocardial wall thickening (WT). In Group 1 (control; $n=7$), a 10-min coronary occlusion was produced followed by reperfusion; WT was monitored until full recovery. In Group 2 ($n=6$), the same occlusion–reperfusion protocol was performed 24 h after inhalation of 1 MAC isoflurane (1.4% in O_2) for 60 min. In Group 3 ($n=5$), the late anti-stunning effect of isoflurane was evaluated following non-selective inhibition of NOS with *N*-nitro-L-arginine (L-NA, 30 mg/kg on 3 days beginning 1 day prior to isoflurane). Expression of eNOS and iNOS protein was measured by Western blotting.
Key Findings: Two to 3 h of reperfusion was required for recovery of WT following isoflurane (Group 2). In contrast, without isoflurane (Group 1), WT remained markedly reduced (30% below baseline) at this time point and required more than 6 h of reperfusion for recovery. Treatment with L-NA (Group 3) did not alter time-course of recovery of WT following isoflurane. Isoflurane caused an increased expression of eNOS, but not of iNOS.
Significance: Isoflurane produced late preconditioning against myocardial stunning. Although this effect was associated with an up-regulation of eNOS, its persistence following L-NA suggested that an increased production of nitric oxide did not play an obligatory role.

Introduction

Volatile anesthetics, such as isoflurane, have been demonstrated to mimic the ability of ischemia to produce both early (Tanaka et al., 2004a) and late (Pagel and Hudetz, 2011) preconditioning (PC), i.e., a second window of protection (SWOP), against myocardial infarction. Our recent study extended the late PC effect of isoflurane to myocardial stunning (Crystal et al., 2012), which is a more mild pathophysiological condition, characterized by persistent contractile dysfunction despite restoration of blood flow and by the absence of irreversible myocardial damage (Bolli and Marban, 1999; Gross et al., 1999).

Late PC requires an up-regulation and/or de novo synthesis of proteins (Baxter and Ferdinandy, 2001). Chiari et al. (2005) demonstrated in rabbits that nitric oxide (NO) derived from endothelial NO synthase (eNOS), but not from inducible NO synthase (iNOS), plays a role in both triggering and mediating isoflurane-induced late PC against myocardial infarction, whereas Wakeno-Takahashi et al. (2005) found in rats that overexpression and activation of iNOS was critical. The involvement of NO, and the relative roles of eNOS and iNOS, in the late anti-stunning effect of isoflurane remain to be determined.

The current study tested the hypothesis that late isoflurane PC against myocardial stunning is dependent upon an increased production of NO. We evaluated the change in expression of iNOS and eNOS in myocardium produced by late isoflurane PC, and attempted to abolish its salutary effect on myocardial stunning with the non-selective NOS inhibitor, *N*-nitro-L-arginine (L-NA). A chronically instrumented, conscious dog model was used to avoid the complications of background anesthesia and acute surgical stress (Kim et al., 1997, 2007).

Materials and methods

The study was conducted after approval from the Institutional Animal Care Committee. The animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, Revised 1996).

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Canine preparation

Twenty mongrel dogs of either sex weighing 20 ± 3 kg underwent anesthesia induction with sodium thiopental (15 mg/kg, iv) and maintenance with isoflurane (0.5%–2.0% in O_2). The animals were instrumented under sterile conditions to measure left circumflex coronary blood flow, and global and regional myocardial function as described in detail previously (Kim et al., 1997, 2007). This included left ventricular (LV) pressure gauges to measure LV pressure, ultrasonic crystals to measure regional wall thickening (WT), and catheters to measure aortic and left atrial pressures. In the posterior region, the crystal pair was implanted in the center of the area at risk, as delineated by epicardial cyanosis during a transient coronary artery occlusion (CAO). A second crystal pair was implanted in the anterior region to provide control measurements for WT. At least 10 days were allowed for recovery from surgery before initiating the experimental protocols. Hemodynamic variables were recorded on a multiple-channel thermal strip chart (Astro-Med Inc., West Warwick, RI).

Experimental protocols

The dogs in Group 1 ($n = 7$) provided a control myocardial stunning response, i.e., without isoflurane PC. After obtaining pre-occlusion baseline measurements of hemodynamic variables, the animal received a bolus injection of morphine sulfate (0.2 mg/kg, im). Myocardial ischemia was produced by inflating a hydraulic occluder. The CAO was maintained for 10 min, which has been demonstrated previously to produce myocardial stunning without evidence of infarction (Kim et al., 2007). Deflation of the coronary occluder was performed gradually to avoid ventricular fibrillation. A bolus injection of lidocaine (1 mg/kg) (Forman et al., 1987) was administered prior to the CAO to prevent cardiac arrhythmias during ischemia/reperfusion. The recovery of WT was evaluated for 24 h following reperfusion.

The dogs of Group 2 ($n = 6$) underwent CAO/reperfusion 24 h after being subjected to isoflurane PC. Measurements of hemodynamic variables were initially obtained in the conscious state. The animal then received thiopental sodium (15 mg/kg, iv) for anesthesia induction. Following tracheal intubation, the animal was administered 1 MAC isoflurane (1.4% in O_2) for 60 min via a ventilator equipped with a calibrated vaporizer. This dose and duration of isoflurane were demonstrated to produce late PC against myocardial stunning in the same canine model (Crystal et al., 2012). The volume and rate of the ventilator were set to maintain arterial CO_2 tension between 35 and 40 mm Hg. A second set of hemodynamic measurements was obtained 50 min into the isoflurane administration. The animal was allowed to recover from the isoflurane administration and returned to its cage. Twenty-four hours later the animal was returned to the laboratory and subjected to the myocardial stunning protocol as described above.

In Group 3 ($n = 5$), the late PC produced by isoflurane was evaluated in the presence of L-NA. L-NA (30 mg/kg/day IV) was administered on three consecutive days to produce a prolonged inhibition of NO production (Kudej et al., 2000), encompassing the triggering and mediation phases of isoflurane late PC; 24 h before the administration of isoflurane, 1 h before the administration of isoflurane, and 1 h before the CAO and subsequent reperfusion. The prolonged effectiveness of the L-NA dose was evaluated by comparing the change in the peak increase in coronary vascular conductance (CVC) following an intra-atrial injection of the endothelial dependent vasodilator, bradykinin (0.05 μg/kg), and the endothelium-independent vasodilator, sodium nitroprusside (2.5 μg/kg) before the initial L-NA administration and 24 h later. CVC was calculated by dividing CBF by mean aortic pressure at the time of the flow measurement.

In vitro studies

After completion of the in vivo study, the dog was anesthetized with pentobarbital sodium (30 mg/kg, iv), its chest was opened, and the heart was rapidly excised and placed in ice-cold saline.

Western blot analysis

Samples of myocardium were obtained from the LV in both Group 1 (control) and Group 2 (isoflurane PC) for protein analysis. Only samples from the anterior region were used to avoid the influence of ischemia–reperfusion on the findings. The tissue was homogenized in ice-cold lysis buffer as described previously (Kim et al., 2007). The primary anti-eNOS and anti-iNOS (Sigma Aldrich) were used. Blots for eNOS and iNOS were incubated overnight at 4 °C temperature with a 1:500 dilution of rabbit anti-eNOS and anti-iNOS, respectively in TBS buffer saline containing 0.1% Tween-20 and 5% nonfat milk. Previous studies have demonstrated that eNOS is a substrate for Akt and that enhanced eNOS function may be associated with activation of the Akt-phosphatidylinositol 3′-kinase (PI3 kinase) signaling cascade (Dimmeler et al., 1999; Fulton et al., 1999). Thus, expression of Akt was also determined. The primary anti-Akt antibody was used and incubated overnight at 4 °C with a 1:500 dilution of rabbit anti-Akt antibody. Primary antibodies were detected using anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma Aldrich) and horseradish peroxidase chemiluminescence detection kit (SuperSignal-Pico, Pierce). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. All Western blot exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by densitometry (ImageQuant 5.2).

Data analysis and statistics

A power analysis based on the improved recovery of contractile function following late ischemic PC in the same conscious canine model (Kim et al., 2007) indicated that a sample size of 4 provided 90% power at a two-sided 0.05 significance level. Statistical significance was assessed using an analysis of variance for repeated measures combined with the Student–Newman–Keuls test for post hoc analysis, and the Student's t test for paired and unpaired samples, as appropriate (Zar, 1974). A value of $P < 0.05$ was considered statistically significant. Data are presented as mean ± SD.

Results

Of the 20 dogs initially instrumented, two were excluded because of equipment failure or evidence of collateral blood flow (recovering wall thickening during CAO).

Table 1 presents values for hemodynamic variables during the CAO and 1 h into the reperfusion period for the control group (no preconditioning) and following isoflurane PC in the absence and presence of L-NA. In all three groups, the CAO reduced CBF to zero, and converted posterior WT (ischemic zone) to paradoxical wall thinning; anterior WT was unaffected. Mean aortic pressure, LV end-diastolic pressure, and heart rate were increased. Hemodynamic variables, including CBF, recovered after 1 h of reperfusion. Fig. 1 compares the recovery of WT over the course of the reperfusion period for the three groups. At 1, 2, 3, and 6 h following reperfusion, recovery of WT with isoflurane PC was greater than the control response (Group 2 vs. Group 1). With isoflurane PC, WT returned completely to the baseline value within 2–3 h of reperfusion, whereas, in the absence of isoflurane PC, WT remained markedly reduced (approximately 30% below the baseline value) at the same time point, and required more than 6 h of reperfusion to completely recover. L-NA did not impair the ability of isoflurane PC to accelerate post-ischemic recovery of WT (Group 2 vs. Group 3).