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Flumazenil Mimics whereas Midazolam Abolishes Ischemic Preconditioning in a Rabbit Heart Model of Ischemia–Reperfusion

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Background: **The goal of the current study was to assess the effects of flumazenil, a benzodiazepine receptor antagonist, in limiting infarct size and in reducing hydroxyl free radical production.**

Methods: **After intravenous salicylate (100 mg/kg) administration, rabbits were subjected to 40 min of regional myocardial ischemia and 2 h of reperfusion. In one group, flumazenil (0.05 mg/kg) and, in another, midazolam (0.05 mg/kg) was administered 15 min before 40 min of ischemia. Ischemic preconditioning (IP) was elicited by 5 min of ischemia followed by 10 min of reperfusion (before the 40-min ischemia period). In two other groups, midazolam was added to flumazenil and IP. Infarct size was determined using triphenyl tetrazolium chloride staining. The authors quantified the hydroxyl-mediated conversion of salicylate to its 2,3- and 2,5-dihydroxybenzoate derivatives during reperfusion by high-performance liquid chromatography coupled with electrochemical detection. Results are expressed as mean ± SEM.**

Results: **Flumazenil, like IP, significantly decreased infarct size (23 ± 4 and 22 ± 5%, respectively, *vs.* 57 ± 6% in control group; $P < 0.01$). Midazolam inhibited the effects of flumazenil and IP. Flumazenil and IP significantly limited the increase in the normalized concentrations of 2,3- and 2,5-dihydroxybenzoic acids. With midazolam, however, the increase was comparable to that of the control group. 5-Hydroxydecanoate, a selective mitochondrial adenosine triphosphate–sensitive K^+ channel blocker, given with flumazenil, abolished the protection obtained with the latter.**

Conclusions: **Flumazenil mimics preconditioning to decrease infarct size and hydroxyl radical production during reperfusion. Midazolam, however, abolishes these effects. Blockade of benzodiazepine receptors is upstream to the mitochondrial adenosine triphosphate–sensitive K^+ channels in the preconditioning cascade.**

REPEATED brief episodes of ischemia, not long enough to cause tissue necrosis, can render the heart more resistant to ischemic injury.[1] The term *ischemic preconditioning* is used to describe the effect of brief coronary occlusions on infarct size produced by subsequent prolonged occlusion. A number of anesthetic drugs have been found either to accentuate ischemic preconditioning or to directly cause pharmacologically mediated preconditioning, without the deleterious effects of preconditioning ischemia.[2–5]

Benzodiazepines are anesthetic agents widely used for patients undergoing cardiac surgery. Flumazenil is a benzodiazepine receptor antagonist. It is used clinically to reverse the effects of benzodiazepines after conscious sedation and general anesthesia and to reverse the effects of benzodiazepine overdose. Zhang and Yao[6] demonstrated that flumazenil was able to mimic preconditioning by reducing cell death in an *in vitro* ischemia–reperfusion model of cardiomyocytes. Flumazenil generated the same magnitude and pattern of reactive oxygen species (ROS) signals as did ischemic preconditioning. Flumazenil-induced protection was abolished by the selective mitochondrial adenosine triphosphate–sensitive K^+ (K_{ATP}) channel blocker 5-hydroxydecanoate. Moreover, Zaugg *et al.*[7] showed that midazolam did not affect K_{ATP} channel activity. K_{ATP} channels are important in the trigger phase of ischemic preconditioning.[8] During ischemia, opening of the K_{ATP} channels benefits the heart, possibly by reducing Ca^{2+} influx through voltage-operated calcium channels, thus slowing adenosine triphosphate depletion and decreasing calcium-induced toxicity. In addition, Leducq *et al.*[9] showed that the specific peripheral benzodiazepine receptor antagonist SSR180575 reduced infarct size and improved cardiac function in various myocardial infarction models.

With reperfusion or reoxygenation, oxygen free radicals are generated.[10] In contrast to their beneficial effect of starting the preconditioning cascade, a large number of studies have demonstrated that oxygen free radicals play a detrimental role in the pathogenesis of reperfusion injury, both directly by damaging membranes and enzymes, and indirectly, by initiating the inflammatory process.[11]

Recently, we have shown that ischemic preconditioning decreased the hydroxyl free radical production in a rabbit model of myocardial ischemia and reperfusion.[12]

The current study was performed using an *in vivo* rabbit model to evaluate whether the action of flumazenil mimics ischemic preconditioning by limiting both

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infarct size and oxygen free radical production. If flumazenil does have such an action, this may add a new and potent means of pharmacologic myocardial preconditioning that could be beneficial for patients with ischemic heart disease. We also assessed whether midazolam, a benzodiazepine commonly used for anesthesia of cardiac patients, abolished the protective effect of ischemic preconditioning. Another goal of the study was to determine the position of the benzodiazepine receptors in the preconditioning cascade.

Materials and Methods

All experiments were conducted following the approval of the Institutional Committee for Animal Care and Laboratory Use (Hadassah-Hebrew University Medical Center, Jerusalem, Israel). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals*.[13]

General Preparation

New Zealand white rabbits weighing between 2.5 and 3.5 kg were initially premedicated with an intramuscular injection of a ketamine (50 mg/ml)–xylazine (10 mg/ml) solution at a volume of 0.6 ml/kg body weight. They were then anesthetized with intravenous pentobarbital (30 mg/kg), administered *via* a 20-gauge intravenous cannula into a marginal ear vein. Hetastarch, 5 $ml \cdot kg^{-1} \cdot h^{-1}$, was infused continuously *via* the intravenous cannula. Anesthesia was maintained during the experiment by pentobarbital supplements as needed (according to pedal and palpebral reflexes). Midazolam and flumazenil were used according to the study protocol. Neuromuscular blockers were not administered in order to assess anesthetic depth. The neck was opened with a ventral midline incision, and a tracheostomy was performed. The rabbits' lungs were mechanically ventilated with positive-pressure ventilation and an inspiratory fraction of oxygen of 1.0. The ventilation rate was 30–35 breaths/min, and tidal volume was approximately 15 ml/kg. The respiratory rate was adjusted to keep the arterial pH between 7.35 and 7.45. End-expiratory carbon dioxide tension was monitored continuously. A 22-gauge catheter filled with heparinized saline was placed in a carotid artery for blood pressure monitoring and blood sampling. Core body temperature was measured *via* a rectal temperature probe and maintained at 38.5° ± 0.3° (normothermic for rabbits) with radiant heat and a warming blanket. Needle electrodes were inserted subcutaneously in a lead II configuration to enable recording of an electrocardiogram to determine heart rate and help confirm the occurrence of ischemia (ST-segment elevation) and reperfusion of the myocardium distal to the coronary occlusion. A left thoracotomy was performed in the fourth intercostal space and a 4-0 silk suture was passed around a prominent branch of the left coronary artery (approximately halfway between the apex and the base) and threaded through a small vinyl tube to form a snare. Coronary artery occlusion was achieved by tightening the snare around the coronary artery. Regional epicardial cyanosis and ST-segment elevation in the electrocardiogram confirmed myocardial ischemia. Reperfusion was achieved by releasing the snare and was confirmed by visual observation of reactive hyperemia. Ventricular fibrillation, if it occurred, was reversed using direct mechanical stimulation: An index finger was flicked directly against the right ventricle side of the fibrillating heart one to three times to achieve defibrillation. Failure to convert to an organized rhythm after three attempts was defined as intractable fibrillation.

Experimental Protocol

Before a 30-min stabilization period, all animals were given salicylate (100 mg/kg) intravenously. Salicylate is a highly effective hydroxyl free radical scavenger that, upon scavenging $^{\cdot}OH$, forms 2,3- and 2,5- dihydroxybenzoic acid (DHBA) by hydroxylation. Although the 2,3-DHBA is a direct product of the reaction of hydroxyl radicals with salicylate, the formation of 2,5-DHBA from salicylate occurs by two independent routes: direct trapping of $^{\cdot}OH$ and biotransformation mediated by the cytochrome P-450 system.[14] All animals underwent 40 min of regional ischemia followed by 2 h of reperfusion. Preconditioning was elicited by 5 min of coronary occlusion followed by 10 min of reperfusion, beginning 15 min before the period of prolonged coronary occlusion. Rabbits were randomly assigned to the following groups (fig. 1): control group (ischemia and reperfusion without further intervention) (C, n = 10); ischemic preconditioning group (IP, n = 10); flumazenil group (F, n = 10), midazolam group (M, n = 10), or midazolam plus ischemic preconditioning group (M + IP, n = 10). Flumazenil (0.05 mg/kg) or midazolam (0.05 mg/kg) (these doses were chosen because they correspond to therapeutic doses) were administered intravenously 15 min before the 40-min ischemia. In the M + IP group, midazolam (0.05 mg/kg) was given 15 min before the short ischemia (*i.e.*, 30 min before the 40-min ischemia). An additional group was added to determine whether the benzodiazepine receptor blocking effect of flumazenil is involved in the mechanism of flumazenil-induced preconditioning: flumazenil plus midazolam group (F + M, n = 10). Flumazenil (0.05 mg/kg) was given 15 min before the administration of midazolam (0.05 mg/kg) and the beginning of the 40-min ischemia. To determine where the benzodiazepine receptors influence the preconditioning cascade, 5-hydroxydecanoate (5-HD, 5 mg/kg), a selective mitochondrial K_{ATP} channel antagonist, was administered with flumazenil: F + 5-HD group (n = 10). To rule out the possibility that 5-HD itself may influence the

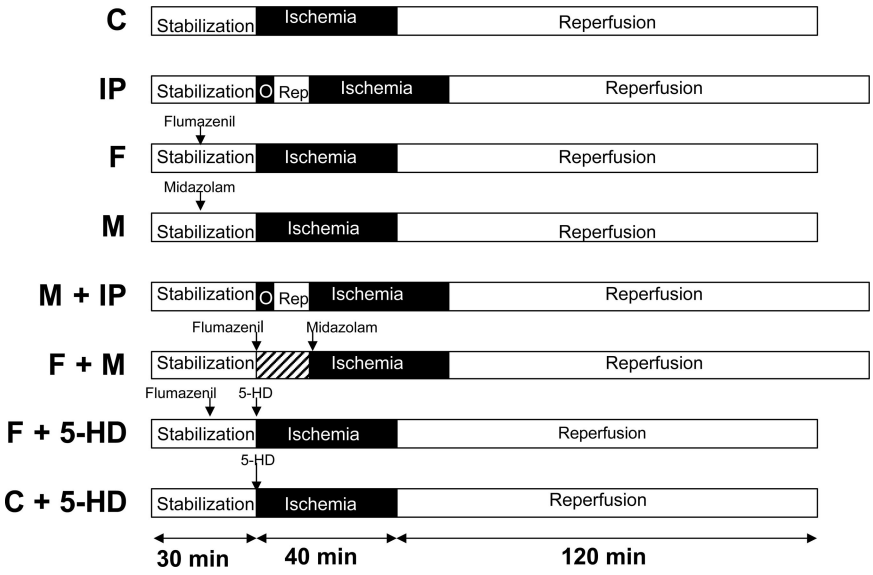

Fig. 1. Schematic illustration of the experimental protocol used. C = control; C + 5-HD = control plus 5-hydroxydecanoate; F = flumazenil; F + 5-HD = flumazenil plus 5-hydroxydecanoate; F + M = flumazenil plus midazolam; IP = ischemic preconditioning; M = midazolam; M + IP = midazolam plus ischemic preconditioning; O = occlusion (5 min); Rep = reperfusion (10 min).

generation of ROS during reperfusion, another control group in which 5-HD was administered before ischemia and reperfusion was also included (C + 5-HD, n = 6). In a third control group (n = 6), 5-HD was given to sham-operated rabbits to rule out any possible direct effect of this agent on the baseline levels and production of ROS in nonischemic hearts (data not shown). Arterial pressure, heart rate, and temperature were recorded continuously. Blood samples for hydroxyl free radical measurements were obtained as follows: at the beginning of the experiment (baseline); at 20 and 40 min of occlusion; every 2 min in the first 10 min of reperfusion; and thereafter at 20, 30, 60, 90, and 120 min of reperfusion. In addition, in group M + IP, blood samples for salicylate and hydroxyl radicals were also collected at the end of the first coronary occlusion and after 5 and 10 min during the first reperfusion period.

We quantified the level of ˙OH-mediated conversion of salicylate to its dihydroxybenzoate derivatives by high-performance liquid chromatography coupled with electrochemical detection.[15] The yield of DHBA derivatives depends on both the flux of the generated hydroxyl radicals and the concentration of salicylate. The values of DHBA derivatives were normalized to account for the difference in salicylate concentration at different time points. Variations in salicylate concentration are attributed to its metabolism and renal clearance. The concentrations of hydroxyl radicals generated in the heart are expressed as the ratio between DHBA (ng) and salicylate (μg).

Determination of Infarct Size and Area at Risk

At the end of the experimental protocol, hearts were excised, mounted on a Langendorff apparatus, and perfused with physiologically buffered saline at 100 cm H_2O for 1 min to wash out intravascular blood. The coronary artery was reoccluded, and 0.2% methylene blue was infused into the aortic root to label the normally perfused zone with deep blue color, thereby delineating the risk zone as a nonstained area. The hearts were then removed from the Langendorff apparatus, trimmed of atria and great vessels, weighed, and frozen (in a cold chamber at a temperature of −18°). Hearts were then cut into 2-mm transverse slices. The slices were incubated in 1% 2,3,5-triphenyl tetrazolium chloride in pH 7.4 buffer for 20 min at 37°. The slices were then placed in 10% neutral buffered formalin for 10 min to increase the contrast between stained and nonstained tissue. Because triphenyl tetrazolium chloride stains viable tissue a deep red color, nonstained tissue was presumed to be infarcted. Slices were then photographed, and risk and infarct areas in each slice were measured by computed planimetry. The mass-weighted average of the ratio of infarct area to the area at risk of the ventricle from each slice was determined (percent infarction).

Quantification of Hydroxyl Radicals by Salicylate

Dihydroxybenzoic acid levels were identified and measured by high-performance liquid chromatography coupled with electrochemical detection using a Varian 5000 liquid chromatograph (Varian Medical Systems, Palo Alto, CA), equipped with a Rheodyne 7125 sample injector (20-μl loop; Rheodyne LLC, Rohnert Park, CA). The column used for separation of salicylate and DHBA was a 25 cm × 4 mm Li Chrospher 100 RP-18, 5 μm (E-Merck, Darmstadt, Germany). The mobile phase contained 0.0 3M citric acid, 0.0 3M acetic acid, 0.2 g/l sodium azide, and 2× methanol. The mobile phase was titrated with solid NaOH to pH 3, followed by titration with CH_3COONa to a final pH of 3.6. The flow rate was 1 ml/min. The system was equipped with two detectors in series. Salicylate was identified and measured fluorometrically using a FD-300 model fluorescence detector (Spectrovision, Chelmsford, MA) using excitation and emission wave lengths of 300 and 412 nm, respectively.

DHBA derivatives were quantified using an electrochemical amperometric detector (model 4A; Bioanalytical Systems, West Lafayette, IN), with a plastic cell equipped with a glass carbon electrode operated at +0.80 V, using an Ag–AgCl reference electrode. The signals from the detector were acquired on an EZChrome data acquisition and handling system (EZChrome Elite; Scientific Software, San Ramon, CA) and subsequently processed.

Statistical Analysis

Hemodynamic data over time within each group were analyzed using analysis of variance with repeated measures on one factor. Differences in hemodynamics between groups were analyzed using analysis of variance with the Tukey *post hoc* test. Incidence of ventricular fibrillation was analyzed with a Kruskal-Wallis test. Intergroup comparisons for infarct sizes were made with one-way analysis of variance, and group differences were detected with Tukey *post hoc* test. Differences between the study groups in DHBA levels were assessed by analysis of variance with the Tukey *post hoc* testing. Statistical calculations were performed using SPSS 10.0 for Windows software (SPSS Inc., Chicago, IL). Data are expressed as mean ± SEM and significance was assumed for $P < 0.05$.

Results

A total of 82 rabbits were studied. The results from 71 animals contributed to the final data set: 8 control, 9 ischemic preconditioning, 9 flumazenil, 8 midazolam, 9 midazolam plus ischemic preconditioning, 9 flumazenil plus midazolam, 9 flumazenil plus 5-HD, and 5 control plus 5-HD–treated rabbits and 6 sham-operated animals. The remaining 11 animals were excluded for technical reasons: accidental release of the snare during the ischemia period (n = 3) or intractable ventricular fibrillation (n = 8). The incidence of refractory ventricular fibrillation was not significantly different among groups (2/10 C, 1/10 IP, 0/10 F, 1/10 M, 1/10 M + IP, 1/10 F + M, 1/10 F + 5-HD, 1/6 C + 5-HD, 0/6 sham-operated animals; $P = 0.35$ with Kruskal-Wallis test).

There was a trend for mean arterial pressure to decrease over time within all groups. Heart rate was similar in all groups, as was the rate–pressure product; therefore, differences in myocardial infarct size between groups could not be attributed to decrease in blood pressure or differences in heart rates. Hemodynamic data are shown in table 1.

The area at risk–to–left ventricular mass ratio did not differ significantly among the groups (51 ± 2% in group

Table 1. Systemic Hemodynamics

			Coronary Occlusion		Reperfusion			
	No.	Baseline	20 min	40 min	4 min	10 min	20 min	30 min
HR, beats/min								
C	8	251 ± 6	239 ± 4	260 ± 8	270± 12	238 ± 5	271 ± 6	277 ± 9
IP	9	235 ± 4	241 ± 11	266 ± 7	246 ± 8	236 ± 3	241 ± 7	270 ± 6
F	9	243 ± 7	246 ± 8	265 ± 3	249 ± 12	235 ± 8	242 ± 4	275 ± 7
M	8	247 ± 5	240 ± 9	262 ± 5	257 ± 11	237 ± 6	247 ± 3	274 ± 6
M + IP	9	250 ± 3	243 ± 7	264 ± 6	268 ± 8	235 ± 6	267 ± 5	272 ± 6
F + M	9	249 ± 2	239 ± 5	264 ± 5	272 ± 6	236 ± 5	268 ± 5	275 ± 6
F + 5-HD	9	248 ± 4	239 ± 5	265 ± 5	253 ± 7	237 ± 4	260 ± 6	275 ± 5
C + 5-HD	5	250 ± 4	240 ± 3	262 ± 5	266 ± 6	238 ± 3	268 ± 5	276 ± 4
MAP, mmHg								
C	8	84 ± 3	71 ± 5	69 ± 9	66 ± 7	65 ± 4	65 ± 2	68 ± 4
IP	9	90 ± 6	72 ± 7	73 ± 5	71 ± 3	69 ± 6	68 ± 7	71 ± 6
F	9	87 ± 1	71 ± 4	70 ± 6	72 ± 4	67 ± 5	65 ± 4	72 ± 7
M	8	86 ± 5	70 ± 3	71 ± 7	69 ± 5	68 ± 4	66 ± 5	70 ± 2
M + IP	9	88 ± 4	71 ± 5	70 ± 5	67 ± 4	66 ± 5	65 ± 6	69 ± 3
F + M	9	86 ± 4	72 ± 6	68 ± 5	70 ± 5	68 ± 4	69 ± 5	70 ± 4
F + 5-HD	9	87 ± 3	72 ± 4	69 ± 7	68 ± 5	67 ± 6	64 ± 5	70 ± 5
C + 5-HD	5	85 ± 4	71 ± 5	70 ± 4	66 ± 6	64 ± 6	65 ± 3	69 ± 4
RPP, $min^{-1} \cdot mmHg \cdot 10^3$								
C	8	21.1 ± 0.8	17.0 ± 1.0	17.9 ± 1.3	17.9 ± 0.7	15.5 ± 0.6	17.6 ± 0.5	18.1 ± 0.7
IP	9	21.1 ± 1.1	17.4 ± 0.7	19.6 ± 0.4	17.5 ± 1.4	16.3 ± 0.4	16.5 ± 1.1	19.2 ± 0.9
F	9	21.1 ± 0.9	17.5 ± 1.1	18.6 ± 0.7	17.9 ± 0.6	15.7 ± 0.7	15.7 ± 0.7	19.8 ± 0.6
M	8	21.2 ± 1.0	16.8 ± 0.8	18.6 ± 0.5	17.7 ± 0.5	16.1 ± 0.4	16.3 ± 0.8	19.2 ± 0.7
M + IP	9	22.0 ± 1.1	17.3 ± 1.2	18.5 ± 0.6	17.9 ± 1.2	15.5 ± 0.5	17.4 ± 0.6	18.8 ± 0.6
F + M	9	21.4 ± 0.7	17.3 ± 1.1	18.0 ± 0.8	18.2 ± 0.7	16.0 ± 0.4	18.4 ± 0.6	19.2 ± 0.5
F + 5-HD	9	21.6 ± 0.8	17.2 ± 0.9	18.3 ± 0.4	17.2 ± 0.7	15.9 ± 0.8	16.6 ± 0.9	19.3± 0.8
C + 5-HD	5	21.3 ± 1.0	17.0 ± 1.1	18.3 ± 0.7	17.6 ± 0.8	15.2 ± 0.5	17.5 ± 0.6	19.0 ± 0.9

Data are presented as mean ± SEM.

Baseline = at the end of the 30-min stabilization period; C = control; C + 5-HD= control plus 5-hydroxydecanoate; F = flumazenil; F + 5-HD = flumazenil plus 5-hydroxydecanoate; F + M= flumazenil plus midazolam; HR = heart rate; IP = ischemic preconditioning; M = midazolam; M + IP = midazolam plus ischemic preconditioning; MAP = mean arterial pressure; RPP = rate–pressure product.

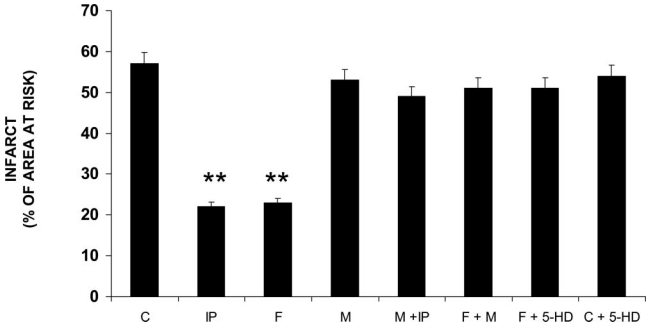

Fig. 2. Myocardial infarct size expressed as a percentage of the left ventricular area at risk. ** Significantly ($P < 0.01$) different from control. C = control; C + 5-HD = control plus 5-hydroxydecanoate; F = flumazenil; F + 5-HD = flumazenil plus 5-hydroxydecanoate; F + M = flumazenil plus midazolam; IP = ischemic preconditioning; M = midazolam; M + IP = midazolam plus ischemic preconditioning.

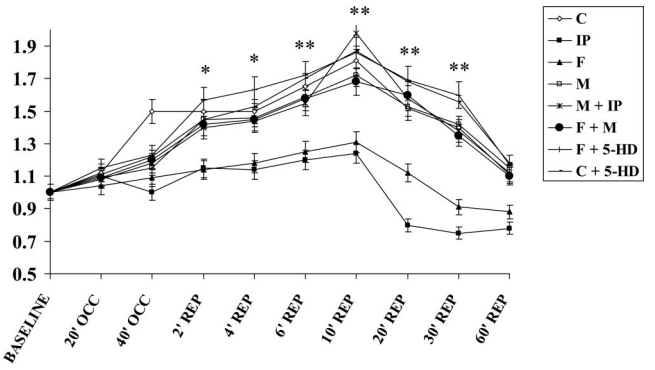

Fig. 3. Mean normalized concentrations of 2,3-dihydroxybenzoic acids (DHBA) (nanograms DHBA/micrograms salicylate [SAL]) in the blood of rabbits exposed to 40 min of regional ischemia (occlusion of a prominent branch of the left coronary artery). The asterisks denote a significant difference between control (C, n = 8), control plus 5-hydroxydecanoate (C + 5-HD, n = 5), midazolam (M, n = 8), midazolam plus ischemic preconditioning (M + IP, n = 9), flumazenil plus midazolam (F + M, n = 9), flumazenil plus 5-hydroxydecanoate (F + 5-HD, n = 9), and ischemic preconditioning (IP, n = 9) and flumazenil (F, n = 8) experiments. * $P < 0.05$. ** $P < 0.01$. OCC = occlusion; REP = reperfusion.

C, 51 ± 1% in group IP, 52 ± 1% in group F, 50 ± 3% in group M, 51 ± 2% in group M + IP, 50 ± 3% in group F + M, 52 ± 2% in group F + 5-HD, and 51 ± 1% in group C + 5-HD). These data suggest that changes in the infarct sizes observed in the different experimental groups cannot be related to the percentage of the left ventricular myocardium that was occluded. In group C, the measured infarct size was 57 ± 6% of the area at risk. Ischemic preconditioning and flumazenil resulted in much smaller infarcts, averaging 22 ± 5 and 23 ± 4%, respectively, of the risk zone ($P < 0.01$). However, the infarct sizes in the M and M + IP groups were similar to that of group C: 53 ± 3 and 49 ± 6%, respectively (P = not significant [NS]), indicating that benzodiazepine receptor activation abolishes ischemic preconditioning. Moreover, when midazolam was added 15 min after flumazenil administration, the cardioprotective effect of flumazenil was abolished (infarct size 51 ± 2%, similar to control). The addition of the K_{ATP} channel blocker 5-HD to flumazenil inhibited the beneficial effect of flumazenil (infarct size of 51 ± 4%, similar to control), suggesting that K_{ATP} channel opening is downstream to benzodiazepine receptor blockade in the mechanism of preconditioning (fig. 2). The measured infarct size in group C + 5-HD was 54 ± 3% of the area at risk (NS compared with group C), thus indicating that 5-HD itself has no direct influence on infarct size (fig. 2).

In the IP and M + IP groups, the level of 2,3 and 2,5-DHBA did not increase significantly after 5 min of index ischemia during the preconditioning period (12% and 9%, respectively, compared with baseline values in group IP [P = NS], and 9% and 8% in group M + IP, respectively [P = NS]). After 5 min of reperfusion following the preconditioning stimulus, there was an increase of 29% ($P < 0.05$) above baseline in 2,3-DHBA. A further 36% increase ($P < 0.05$) above baseline levels was observed after 10 min, at the end of the first reperfusion period. The level of 2,5-DHBA increased by 26% compared with baseline values ($P < 0.05$) at 5 min of reperfusion and increased further to 32% ($P < 0.05$) above baseline values after 10 min. Administration of midazolam attenuated the increase in hydroxyl radical levels in group M + IP during the preconditioning period: The measured normalized values of 2,3-DHBA were 13% (P = NS) and 16% (P = NS) above baseline levels after 5 and 10 min of the first reperfusion period, respectively. Furthermore, as was observed with 2,3-DHBA, midazolam attenuated the increase in the concentration of 2,5-DHBA during preconditioning: The measured value of 2,5-DHBA was only 12% (P = NS) above baseline levels at 5 min of reperfusion and 14% (P = NS) above the baseline values at 10 min.

An acute increase of 50% in normalized 2,3-DHBA compared with baseline values ($P < 0.05$) was already observed in the control group after 2 min of reperfusion. After 10 min of reperfusion, the peak value was measured (81% increase compared with baseline levels; $P < 0.01$; fig. 3). After 10 min of reperfusion, there was only a 24% and 31% increase in the concentration of 2,3-DHBA in the IP and F groups, respectively ($P < 0.01$ compared with control group). However, the administration of midazolam (groups M and M + IP) did not produce this effect: The hydroxyl radical production increased to a degree similar to that seen in the control group (increase of 72% and 68%; P = NS compared with group C). The addition of 5-HD to flumazenil resulted in an increase of 68% of the normalized 2,3-DHBA similar to that of the control group. In group C + 5-HD, there was an increase of 87% in the concentration of the 2,3 derivative (P = NS compared with group C), thus ruling out any possible effect of 5-HD itself on the generation of hydroxyl radicals. Maximal production of 2,5-DHBA occurred in the control group after 4 min of reperfusion: a

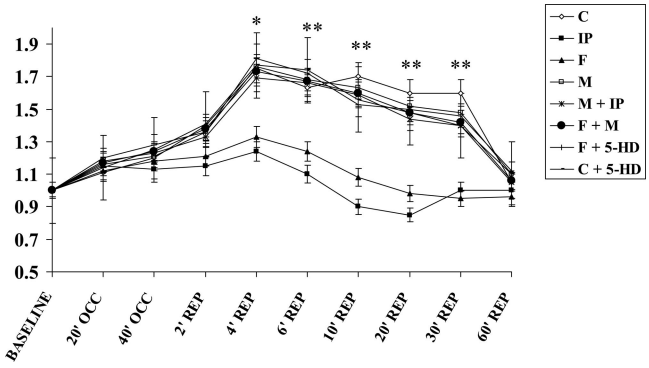

Fig. 4. Mean normalized concentrations of 2,5-dihydroxybenzoic acids (DHBA) (nanograms DHBA/micrograms salicylate [SAL]) in the blood of rabbits exposed to 40 min of regional ischemia (occlusion of a prominent branch of the left coronary artery). The asterisks denote a significant difference between control (C, n = 8), control plus 5-hydroxydecanoate (C + 5-HD, n = 5), midazolam (M, n = 8), midazolam plus ischemic preconditioning (M + IP, n = 9), flumazenil plus midazolam (F + M, n = 9), flumazenil plus 5-hydroxydecanoate (F + 5-HD, n = 9), and ischemic preconditioning (IP, n = 9) and flumazenil (F, n = 8) experiments. * $P < 0.05$. ** $P < 0.01$. OCC = occlusion; REP = reperfusion.

75% increase compared with baseline values ($P < 0.01$; fig. 4). Ischemic preconditioning and flumazenil, however, significantly attenuated this increase to only 24% and 33%, respectively, above baseline values ($P < 0.05$ compared with group C). The increases in 2,5-DHBA in the M (76%), M + IP (69%), and F + 5-HD (81%) groups were comparable with that of the control group (P = NS). As with 2,3-DHBA, 5-HD itself had no effect on the production of 2,5-DHBA. After 4 min of reperfusion, there was an increase of 77% in the level of this derivative compared with baseline levels. This increase was similar to that of the control group (P = NS).

At all time points during reperfusion (up to 30 min), there was a significant difference between the C, M, M + IP, and F + 5-HD groups on one hand, and the IP and F groups on the other hand (figs. 3 and 4).

At 60 min of reperfusion and afterward, there was a return to baseline values in all groups.

In the sham-operated animals, there was no significant difference in the concentration of the DHBA derivatives compared with baseline levels in all time points (data not shown).

Discussion

In this *in vivo* rabbit model of ischemia and reperfusion, flumazenil reduced infarct size and decreased postischemic production of hydroxyl radicals to a degree similar to that produced by ischemic preconditioning. These data thus indicate that flumazenil mimics ischemic preconditioning. Alternately, the benzodiazepine midazolam had the opposite effect; namely, it abolished ischemic preconditioning. Therefore, directly or indirectly, benzodiazepine receptors influence ischemic preconditioning. These findings confirm and extend the previous *in vitro* report by Zhang and Yao,[6] who found that flumazenil reduced cell death in chick cardiomyocytes, despite its discontinuation 10 min before 60-min ischemia. In the current *in vivo* study, flumazenil administered before the ischemic period elicited similar cardioprotective effects. The mechanisms by which flumazenil mediates these actions in cardiomyocytes seem to be identical to those seen with classic ischemic preconditioning, where several parallel endogenous signaling pathways seem to be involved, including adenosine, acetylcholine, K_{ATP} channels, ROS, protein kinase C, and others.[16] Yao *et al.*[17] showed in cultured cardiomyocytes that flumazenil induced cardioprotection by generating ROS. An increase in ROS signals activated protein kinase C and subsequently opened mitochondrial K_{ATP} channels. It has yet to be demonstrated that the same mechanism is involved in the cardioprotection seen in our *in vivo* model.

In contrast to the beneficial effects of ROS and metabolites in triggering ischemic or pharmacologic preconditioning, they have been implicated at being involved in the postischemic reperfusion injury of the heart.[10] ROS cause lipid peroxidation that disrupts membrane function permitting calcium overload to occur.[11] ROS can induce structural alterations in both vascular cells and cardiac myocytes, leading to a decrease in heart contractility and a marked increase in coronary vascular resistance. The current study suggests that a simple procedure, such as flumazenil administration, offers another method to protect the heart by attenuating free radical production during reperfusion and also reducing cell death.

Benzodiazepine effects are mediated primarily *via* the central benzodiazepine receptors located in the central nervous system, but they also bind to other receptors, called peripheral benzodiazepine receptors, located mainly in peripheral tissues such as the heart.[18] Leducq *et al.*[9] have shown that a specific peripheral benzodiazepine receptor antagonist, SSR180575, protected the heart against ischemia–reperfusion injury. Although flumazenil is not a selective peripheral benzodiazepine receptor ligand, but rather a potent antagonist of the central benzodiazepine receptors,[19] we were able to show that it had significant protective cardiac effects. In the current study, however, midazolam, a benzodiazepine receptor agonist, neither limited infarct size nor decreased ROS production during reperfusion after ischemia. When administered before ischemic preconditioning or flumazenil, it inhibited their beneficial effects, suggesting that benzodiazepine receptors may be implicated in their mechanism of cardioprotection. It is of interest that benzodiazepine receptors have been identified in mitochondria.[9,20] During ischemic preconditioning, minute ROS production in the mitochondria occurs, leading to the opening of mitochondrial K_{ATP} channels

and subsequent activation of protein kinases. Furthermore, we showed that selective blockade of the mitochondrial K_{ATP} channels by 5-HD during flumazenil administration abolished the reductions in infarct size and hydroxyl free radical production seen with flumazenil. Therefore, K_{ATP} channel opening is downstream to benzodiazepine receptor inhibition in the flumazenil protective cascade.

This study may have implications in the clinical practice because midazolam is a widely used benzodiazepine in cardiac patients, especially in coronary artery bypass surgery patients. It is of interest that, in the clinical setting, *i.e.*, coronary surgery, midazolam has been associated with a decreased preservation of early postoperative myocardial function (as demonstrated by poorer hemodynamics, higher concentrations of troponin I, and need for inotropic support) compared with the volatile anesthetics sevoflurane or desflurane.[21] Alternately, flumazenil, a benzodiazepine antagonist, has been shown to produce minimal coronary and left ventricular hemodynamic responses in patients with coronary artery disease after reversal of flunitrazepam-induced sedation.[22] Therefore, flumazenil-induced cardioprotection may have a role in clinical situations where the deleterious effects of ischemia–reperfusion are expected, specifically in high-risk patients in whom an ischemic type of preconditioning may further jeopardize diseased myocardium.

References

1. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36
2. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. ANESTHESIOLOGY 1997; 86:699–709
3. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics preconditioning *via* activation of K_{ATP} channels: Reduction of myocardial infarct size with an acute memory phase. ANESTHESIOLOGY 1997; 87:361–70
4. 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
5. Piriou V, Chiari P, Lhuillier F, Bastien O, Loufoua J, Raisky O, David JS, Ovize M, Lehot JJ: Pharmacological preconditioning: Comparison of desflurane, sevoflurane, isoflurane and halothane in rabbit myocardium. Br J Anaesth 2002; 89: 486–91
6. Zhang Q, Yao Z: Flumazenil preconditions cardiomyocytes *via* oxygen radicals and K_{ATP} channels. Am J Physiol Heart Circ Physiol 2000; 279:H1858–63
7. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Garcia C, Schaub MC: Differential effects of anesthetics on mitochondrial K_{ATP} channel activity and cardiomyocyte protection. ANESTHESIOLOGY 2002; 97:15–23
8. Gross GJ, Auchampach JA: Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. Circ Res 1992; 70:223–33
9. Leducq N, Bono F, Sulpice T, Vin V, Janiak P, Le Fur GL, O'Connor SE, Herbert JM: Role of peripheral benzodiazepine receptors in mitochondrial, cellular, and cardiac damage induced by oxidative stress and ischemia-reperfusion. J Pharmacol Exp Ther 2003; 306:828–37
10. Simpson PJ, Lucchesi BR: Free radicals and myocardial ischemia and reperfusion injury. J Lab Clin Med 1987; 110:13–30
11. Opie LH: Reperfusion injury and its pharmacological modification. Circulation 1989; 80:1049–62
12. Raphael J, Drenger B, Rivo J, Berenshtein E, Chevion M, Gozal Y: Ischemic preconditioning decreases the reperfusion-related formation of hydroxyl radicals in a rabbit model of regional myocardial ischemia and reperfusion: The role of K_{ATP} channels. Free Rad Res 2005; 39:747–54
13. Guide for the Care and Use of Laboratory Animals. Bethesda, US National Institutes of Health, revised 1996. NIH publication No. 85-23.
14. Glantz L, Ginosar Y, Chevion M, Gozal Y, Elami A, Navot N, Kitrossky N, Drenger B: Halothane prevents postischemic production of hydroxyl radicals in the canine heart. ANESTHESIOLOGY 1997; 86:440–7
15. Powell SR, Hall D: Use of salicylate as a probe for .OH formation in isolated ischemic rat hearts. Free Radic Biol Med 1990; 9:133–41
16. Tanaka K, Ludwig LM, Kersten JR, Pagel PS, Warltier DC: Mechanisms of cardioprotection by volatile anesthetics. ANESTHESIOLOGY 2004; 100:707–21
17. Yao Z, McPherson BC, Liu H, Shao Z, Li C, Qin Y, Vanden Hoek TL, Becker LB, Shumacker PT: Signal transduction of flumazenil-induced preconditioning in myocytes. Am J Physiol Heart Circ Physiol 2001; 280:H1249–55
18. Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G, Weizman A: Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev 1999; 51:629–50
19. Pisu MG, Papi G, Porcu P, Trapani G, Latrofa A, Biggio G, Serra M: Binding of [3H]CB34, a selective ligand for peripheral benzodiazepine receptors, to rat brain membranes. Eur J Pharmacol 2001; 432:129–34
20. Zhang HY, McPherson BC, Liu H, Baman TS, Rock P, Yao Z: H_2O_2 opens mitochondrial K_{ATP} channels and inhibits GABA receptors *via* protein kinase C-ε in cardiomyocytes. Am J Physiol Heart Circ Physiol 2002; 282:H1395–403
21. De Hert SG, Van der Linden PJ, Cromheeke S, Meeus R, ten Broecke PW, De Blier IG, Stockman BA, Rodrigus IE: Choice of primary anesthetic regimen can influence intensive care unit length of stay after coronary surgery with cardiopulmonary bypass. ANESTHESIOLOGY 2004; 101:9–20
22. Marty J, Nitenberg A, Philip I, Foult JM, Joyon D, Desmonts JM: Coronary and left ventricular hemodynamic responses following reversal of flunitrazepam-induced sedation with flumazenil in patients with coronary artery disease. ANESTHESIOLOGY 1991; 74:71–6