Effects of selective inhibition of cytochrome P-450 ω-hydroxylases and ischemic preconditioning in myocardial protection

Kasem Nithipatikom,1 Michael P. Endsley,1 Jeannine M. Moore,1 Marilyn A. Isbell,1 John R. Falck,2 William B. Campbell,1 and Garrett J. Gross1

1Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin; and 2Departments of Biochemistry and Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas

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Nithipatikom, Kasem, Michael P. Endsley, Jeannine M. Moore, Marilyn A. Isbell, John R. Falck, William B. Campbell, and Garrett J. Gross. Effects of selective inhibition of cytochrome P-450 ω-hydroxylases and ischemic preconditioning in myocardial protection. Am J Physiol Heart Circ Physiol 290: H500–H505, 2006. First published October 7, 2005; doi:10.1152/ajpheart.00918.2005.—Cytochrome P-450 (CYP) ω-hydroxylases and their arachidonic acid (AA) metabolite, 20-hydroxyeicosatetraenoic acid (20-HETE), produce a detrimental effect on ischemia-reperfusion injury in canine hearts, and the inhibition of CYP ω-hydroxylases markedly reduces myocardial infarct size expressed as a percentage of the area at risk (IS/AAR, %). In this study, we demonstrated that a specific CYP ω-hydroxylase inhibitor, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), markedly reduced 20-HETE production during ischemia-reperfusion and reduced myocardial infarct size compared with control [19.5 ± 1.0% (control), 9.6 ± 1.5% (0.40 mg/kg DDMS), 4.0 ± 2.0% (0.81 mg/kg DDMS), P < 0.01]. In addition, 20-hydroxyeicosaa-6(Z),15(Z)-diene acid (20-HEDE, a putative 20-HETE antagonist) significantly reduced myocardial infarct size from control [10.3 ± 1.3% (0.032 mg/kg 20-HEDE) and 5.9 ± 1.9% (0.064 mg/kg 20-HEDE), P < 0.05]. We further demonstrated that one 5-min period of ischemic preconditioning (IPC) reduced infarct size to a similar extent as that observed with the high doses of DDMS and 20-HEDE, and the higher dose of DDMS given simultaneously with IPC augmented the infarct size reduction [9.9 ± 2.8% (IPC) to 2.5 ± 1.4% (0.81 mg/kg DDMS), P < 0.05] to a greater degree than that observed with either treatment alone. These results suggest an important negative role for endogenous CYP ω-hydroxylases and their product, 20-HETE, to exacerbate myocardial injury in canine myocardium. Furthermore, for the first time, this study demonstrates that the effect of IPC and the inhibition of CYP ω-hydroxylase synthesis (DDMS) or its actions (20-HEDE) may have additive effects in protecting the canine heart from ischemia-reperfusion injury.

20-hydroxyeicosaa-6(Z),15(Z)-diene acid; 20-hydroxyeicosatetraenoic acid; ischemia; preconditioning; reperfusion

CAMEINE HEARTS subjected to ischemia-reperfusion injury result in an accumulation of unesterified arachidonic acid (AA) (4, 18). Free AA can be metabolized by cyclooxygenase (COX), lipoygenase (LOX), and cytochrome P-450 (CYP) to many biologically active eicosanoids. Some of these AA metabolites are cardioprotective and some are detrimental. The COX and LOX pathways have been extensively investigated in the myocardium. Recently, the function of the CYP pathways and their metabolites has been investigated in several animal models of ischemia-reperfusion injury (7, 8, 15, 17). Transgenic mice with cardiomyocyte-specific overexpression of CYP2J2 (which synthesizes epoxyeicosatrienoic acids, EETs, from AA) exhibited cardioprotection after 20-min ischemia and 40-min reperfusion through the activation of the mitochondrial ATP-sensitive K⁺ channel and p42/p44 mitogen-activated protein kinase (17). However, nonselective CYP inhibitors such as chloramphenicol, cimetidine, and sulfaphenazole reduced myocardial infarct size, and the reduction of infarct size was attributed to the ability of these compounds to prevent CYP-derived free radical formation during reperfusion (6, 7). The cardioprotective effect of CYP epoxygenases and CYP-derived eicosanoids as well as the deleterious effect of CYP-derived reactive oxygen species (ROS) in myocardial ischemia-reperfusion injury has been recently discussed, and there is strong evidence to support a role for ROS after CYP activation (3).

We previously found high plasma concentrations of CYP metabolites of AA, particularly the CYP ω-hydroxylase metabolite 20-hydroxyeicosatetraenoic acid (20-HETE), during ischemia and after reperfusion in the canine heart (13, 15). Several CYP ω-hydroxy- and hydroxyeicosatetraenoic acid species that produce 20-HETE such as CYP4A1, CYP4A2, and CYP4F present in canine myocardium. Both the nonspecific CYP inhibitor miconazole and the specific CYP ω-hydroxylase inhibitor 17-octadecynoyl acid markedly inhibit 20-HETE production during ischemia-reperfusion and have been shown to reduce myocardial infarct size (8, 15).

Ischemic preconditioning (IPC) was first demonstrated in dog hearts in which 4 multiple brief ischemic episodes (5 min) interspersed with brief periods of reperfusion produced an approximately 70–80% reduction in infarct size from a subsequent sustained ischemic period (12). Later studies indicate that a single episode of brief occlusion is as effective as a series of brief occlusions in protection of the canine heart (9). At the present time, IPC is thought to be the most efficacious cardioprotective intervention yet discovered. However, the magni-
tude of the effect of inhibiting CYP and IPC alone and of combining the two in exacerbating or reducing myocardial ischemia-reperfusion injury has not been compared.

In this study, we investigated the role of a specific CYP ω-hydroxylase inhibitor, N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS), and a putative 20-HETE antagonist, 20-hydroxyeicosapentaenoic acid (20-HEDE) (1, 19), in reducing myocardial infarct size in dogs. We also compared the effects of IPC and DDMS alone or in combination on the magnitude of cardioprotection observed in alleviating myocardial ischemia-reperfusion injury. Finally, a higher dose of 20-HETE than previously used in our laboratory (13, 15) was tested to determine if a further increase in infarct size might be observed.

MATERIALS AND METHODS

Materials. AA, EETs, dihydroxyeicosatetraenoic acids (DHETs), and 20-HETE [for eicosanoid determination by mass spectrometry (MS)] were obtained from Cayman Chemical (Ann Arbor, MI). DDMS, EETs, 20-HEDE, 20-HETE, and 20-[3H2]HETE were synthesized in the laboratory of J. R. Falck. 14,15-[3H2]DHET and [3H]EETs were synthesized in our laboratory (14). C18 Bond Elut solid-phase extraction (SPE) columns were obtained from Varian (Harbor City, CA). All other chemicals and solvents were of analytical or highest purity grades. Distilled, deionized water was used in all experiments.

General preparation of dogs. All experiments conducted in this study were in accordance with the “Position of the American Heart Association on Research and Animal Use” adopted by the American Heart Association and the Guidelines of the Biomedical Resource Center of the Medical College of Wisconsin, and protocols were approved by the Animal Care Committee. The Medical College of Wisconsin is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

The protocol for dog preparation and experiments has been previously described in detail (11). Briefly, adult mongrel dogs of either sex, weighing 18–25 kg, were fasted overnight, anesthetized with the combination of sodium barbital (200 mg/kg) and pentobarbital sodium (15 mg/kg), and ventilated with room air supplemented with 100% oxygen. Body temperature was maintained at 38 ± 1°C with a heating pad. Atelectasis was prevented by maintaining an end-expiratory pressure of 5–7 cmH2O with a trap. Arterial blood pH, PCO2, and PO2 were monitored at selected intervals by an AVL automatic blood gas system and maintained within normal physiological limits (pH 7.35–7.45, PCO2 30–40 mmHg, and PO2 85–120 mmHg) by adjusting the respiration rate and oxygen flow or by intravenous administration of 1.5% sodium bicarbonate if necessary. A flowmeter (Statham 2202) was used to measure left anterior descending coronary artery (LAD) blood flow. A mechanical occluder was placed distal to the flow probe such that there were no branches between the flow probe and the occluder. Hemodynamics, heart rate, and coronary blood flow were monitored and recorded by a polygraph throughout the experiment. The left atrium was cannulated via the appendage for radioactive microsphere injections.

Experimental design. Dogs were sequentially assigned to groups for different treatments. Normally, eight dogs were studied in each group of experiments. At 15 min before the 60-min LAD occlusion period, DDMS (0.40 and 0.81 mg/kg), 20-HEDE (0.032 and 0.064 mg/kg), 20-HETE (0.128 mg/kg), or vehicle was administered by intracoronary infusion for 2–3 min as shown in protocol 1 (Fig. 1). For IPC experimental groups, a brief occlusion of 5 min was performed at 15 min after the administration of drugs (or vehicle) and the subsequent 60-min LAD occlusion (10 min after IPC) as shown in protocol 2 (Fig. 1). In all groups, hemodynamic measurements, blood gas analyses, and myocardial blood flow measurements were performed at baseline and at 30 min into the 60-min occlusion period. After reperfusion, hemodynamics were measured every hour and myocardial blood flow was determined at the end of the 3-h reperfusion period. At the end of the experiment, the hearts were electrically fibrillated, removed, and prepared for infarct size determination and regional myocardial blood flow measurement.

Infarct size determination. Infarct size was determined as previously described (11). Briefly, at the end of the 3-h reperfusion period, the LAD was cannulated. To determine the anatomic area at risk (AAR) and the nonischemic area, 5 ml of Patent blue dye and 5 ml of saline were injected at equal pressure into the left atrium and LAD, respectively. The heart was then immediately fibrillated and removed. The left ventricle was dissected and sliced into serial transverse sections 6–7 mm in width. The nonstained ischemic area and the blue-stained normal area were separated and incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) in 0.1 mol/l phosphate buffer, pH 7.4, at 37°C for 15 min. After incubation overnight in 10% formaldehyde, the noninfarcted and infarcted tissues within the AAR were separated and determined gravimetrically. Infarct size was expressed as a percentage of the AAR.

Regional myocardial blood flow. Regional myocardial blood flow was measured by the radioactive microsphere technique as previously reported (2). Microspheres were administered at 30 min into the 60-min occlusion period and at the end of 3-h of reperfusion. Transmural blood flow was calculated as the weighted average of the subepicardium, midmyocardium, and subendocardium in each region.

Fig. 1. Protocols for drug administration and ischemia-reperfusion of dogs. Protocol 1: drug treatment (Drug), occlusion (Occ), and following reperfusion (Rep). Protocol 2: drug treatment combined with ischemic preconditioning (IPC), occlusion, and following reperfusion. Bottom arrows in both panels represent time points for collecting blood samples for determination of eicosanoids. BL, baseline; DS, drug sample.
Sample collection and preparation of CYP metabolites of AA. Arterial (femoral artery) and venous plasma (great cardiac vein) samples were collected as previously described (13). Briefly, blood was drawn through an 8-F single lumen (100-cm length) catheter. Once the catheter was cleared of residual, stagnant blood, 5 ml of blood was aspirated over a 3- to 5-s period into a chilled sample tube containing heparin (1,000 U/ml) and miconazole (5 × 10⁻⁵ mol/l), mixed thoroughly, and placed in an ice bucket. Blood samples were collected at different times as indicated in protocols 1 and 2 (Fig. 1). The samples were centrifuged at 3,000 g at 0°C for 10 min to separate plasma. Plasma was removed and transferred to a tube, purged with nitrogen gas, capped, and stored at −80°C or extracted.

The internal standards, 1.0 ng each for [²H₈]EETs, [²H₈]DHETs, and 20-[²H₈]HETE, were added to each sample and mixed; then ethanol was added to a final concentration of 15% ethanol, mixed, and centrifuged at 1,500 rpm for 3 min. Plasma samples were extracted by SPE as previously described (14).

Liquid chromatography-electrospray ionization-MS determination of CYP metabolites of AA. CYP metabolites of AA in plasma samples were analyzed by liquid chromatography-electrospray ionization-MS (LC-ESI-MS) (Agilent 1100 LC/MSD, SL Model) as previously described (14). Selected ion monitoring (SIM) in the negative detection mode was used for determination of the CYP metabolites of AA.

Statistical analysis. All values are expressed as means ± SE. Differences between groups in hemodynamics were compared by using a two-way ANOVA. Differences between groups in tissue blood flows, AAR, and infarct size were compared by one-way ANOVA, and differences in concentrations of AA metabolites at various times during occlusion and after reperfusion between treatment groups and the control group were compared by using a two-way repeated-measures ANOVA followed by a Tukey’s post hoc test. Differences between groups were considered significant if P < 0.05.

RESULTS

Regional myocardial blood flow. Transmural blood flows in the nonischemic (left circumflex coronary) and the ischemic (LAD) regions were measured during 60 min of occlusion and after 3 h of reperfusion. There was no significant difference in transmural collateral blood flow or AARs (Figs. 2A and 3A) between groups, indicating that all groups were subjected to similar degrees of ischemia (0.07 ± 0.02, control; 0.11 ± 0.02, high-dose DDMS; 0.08 ± 0.03, 20-HETE; 0.10 ± 0.02, IPC; 0.09 ± 0.01, high-dose DDMS + IPC). Three animals were excluded from data analysis due to a high collateral flow (endocardial > 0.15 ml·min⁻¹·g⁻¹).

Hemodynamic responses. Heart rate, mean arterial blood pressure, and pressure-rate product at baseline between groups or at subsequent times throughout the experiment were not significantly different (data not shown). In the animals where a coronary flow probe was able to be successfully placed around the LAD (n = 3), surprisingly, no decreases in coronary blood flow were noted during or after infusion of 20-HETE as might be expected. All other compounds administered produced increases in coronary blood flow that rapidly disappeared after the end of the drug infusion period (5 min).

Effects of DDMS, 20-HETE, and 20-HETE on myocardial infarct size. Anatomic AAR expressed as a percentage of the left ventricle (AAR/LV) in DDMS-, 20-HETE-, and 20-HETE-treated groups was not significantly different from the control group (Fig. 2A). However, myocardial infarct size (IS) expressed as a percentage of the AAR (IS/AAR) in the treatment groups was significantly different from the control group (Fig. 2B). DDMS at 0.40 mg/kg and 0.81 mg/kg markedly reduced infarct size (9.6 ± 1.5 and 4.0 ± 2.0%, respectively) compared with the control group (19.5 ± 1.0%). 20-HETE at 0.032 mg/kg and 0.064 mg/kg also reduced infarct size to 10.3 ± 1.3 and 5.9 ± 1.9%, respectively. On the other hand, exogenous 20-HETE at 0.128 mg/kg increased infarct size to 31.0 ± 5.6%, respectively.

Effects of IPC and DDMS in cardioprotection. AAR expressed as a percentage of the LV (AAR/LV) in IPC and IPC + DDMS-treated groups was not significantly different from the control group (Fig. 3A). A brief 5-min period of IPC of the heart 10 min before the 60-min occlusion decreased infarct size to 9.9 ± 2.8%, similar to previous results obtained in our laboratory (16). More importantly, DDMS at 0.40 mg/kg administered 15 min before IPC did not further reduce the infarct size (6.6 ± 1.9%), but DDMS at 0.81 mg/kg reduced the infarct size to 2.5 ± 1.4% (Fig. 3B).

Fig. 2. Area at risk (AAR) expressed as a percentage of left ventricle (LV; A) and infarct size (IS) expressed as a percentage of AAR (B) for the following treatments: DDMS(1) and DDMS(2), 0.40 mg/kg and 0.81 mg/kg N-methylsulfonyl-12,12-Dibromododec-11-enamide, respectively; 20-HETE, 0.128 mg/kg 20-hydroxyeicosatetraenoic acid; 20-HDE(1) and 20-HDE(2), 0.032 and 0.064 mg/kg 20-hydroxyeicosacosa-6(Z),15(Z)-dienoic acid, respectively. AAR/LV is not significantly different among groups. DDMS and 20-HETE significantly reduced infarct size (B). On the other hand, 20-HETE significantly increased infarct size. Values are means ± SE; n = 8 per group. *Significantly lower than control (P < 0.01); # significantly higher than control (P < 0.05).
Plasma CYP metabolites of AA. 20-HETE was detected as the major CYP metabolite of AA in dog coronary venous plasma collected during ischemia and reperfusion. 11,12- and 14,15-DHET were also detected at much lower concentration than 20-HETE, similar to previous study (15). Again, EETs were not detectable in these venous plasma samples, indicating that EETs may be rapidly hydrolyzed to DHETs. The plasma concentrations of 11,12- and 14,15-DHET did not significantly change in these treatment groups from the control. Administration of a specific CYP \( \omega \)-hydroxylase inhibitor, DDMS, significantly reduced the plasma concentration of 20-HETE (Fig. 4A). Intriguingly, 20-HEDE, a putative 20-HETE antagonist, also reduced the plasma concentrations of 20-HETE (Fig. 4A).

IPC significantly reduced the plasma concentration of 20-HETE during the reperfusion compared with the control dogs (Fig. 4B) while the plasma concentrations of 11,12-DHET and 14,15-DHET were not affected (data not shown). DDMS at 0.40 mg/kg did not further reduce the plasma concentration of 20-HETE from the IPC group; however, DDMS at 0.81 mg/kg decreased the plasma concentration of 20-HETE after drug administration and IPC. It would have been interesting to have combined DDMS and 20-HEDE because their actions to reduce 20-HETE synthesis and 20-HETE actions would also be expected to result in a synergistic effect on IS/AAR similar to or greater than that observed with DDMS and IPC.

**DISCUSSION**

We have previously demonstrated that CYP \( \omega \)-hydroxylases and their AA metabolite, 20-HETE, have a significant detri-
mental role in enhancing myocardial ischemia-reperfusion injury in the canine and rat heart. In this study, we demonstrate that a specific CYP ω-hydroxylase inhibitor, DDMS, markedly reduces the plasma concentration of 20-HETE and reduces infarct size in canine hearts subjected to a 60-min occlusion period followed by 3h of reperfusion. The reduction of infarct size was also observed with the exogenous administration of a putative 20-HETE receptor antagonist, 20-HEDE, administered 15 min before occlusion. The exogenous administration of a high dose of 20-HETE 15 min before occlusion increased infarct size similar to that previously observed with a fourfold lower dose of 20-HETE (13, 15). Although it may seem surprising that a 5-min infusion of 20-HETE would produce a long-lasting effect to increase infarct size, these results are consistent with those recently published by Miyata et al. (10) in which they found that intracardiot artery injection of 20-HETE produced a cerebral infarct similar in severity to that observed in an ischemic stroke model in rats. Taken together, these results suggest that activation of CYP ω-hydroxylases and administration of exogenous 20-HETE have significant detrimental effects on the canine myocardium during ischemia and/or after reperfusion.

Several recent studies indicate that CYP epoxygenases and their AA metabolites, the EETs, are cardioprotective (5, 17). In plasma samples, only 11,12- and 14,15-DHET but not the parent compounds were detected, indicating that the EETs were rapidly hydrolyzed. The plasma concentrations of DHETs were much lower than the plasma concentration of 20-HETE. Based on these results, it is not known whether the increase in EETs or the decrease in 20-HETE is more significant in producing the cardioprotective observed. Most likely, it is the combination of these two approaches that may maximize the contribution of the CYP pathway in distinguishing the extent of myocardial injury in this model. A putative 20-HETE antagonist, 20-HEDE, also significantly reduced infarct size. Although purported to be a 20-HETE receptor antagonist, the mechanism by which 20-HEDE protects the myocardium during ischemia-reperfusion-injury is not well established. Surprisingly, 20-HEDE produced a decrease in the plasma concentration of 20-HETE. The mechanism for the decrease of 20-HETE by 20-HEDE is not known. However, incubation of heart and kidney microsomes with [14C]AA in the absence and presence of 20-HEDE indicated that 20-HEDE did not directly inhibit the production of 20-HETE. Thus it is speculated that the reduction of the plasma 20-HETE concentration in vivo might be from a different mechanism. Interestingly, IPC also resulted in a significant reduction in 20-HETE concentrations in coronary venous blood similar to the reduction produced by DDMS and 20-HEDE. This is the first study in which the effect of IPC on the CYP pathway has been studied. However, the mechanism responsible for this effect of IPC is not clear and requires further study. It may be that numerous cardioprotective interventions reduce the activation of the CYP ω-hydroxylase pathway in general, or it may be an effect unique to selective enzyme inhibitors and IPC.

Interestingly, the administration of DDMS in conjunction with IPC resulted in a larger reduction in infarct size than either intervention separately. Unfortunately, the effect of these two interventions in combination on 20-HETE concentrations in coronary venous blood was not greater than that seen with either intervention alone so it appears that a further reduction in 20-HETE is not responsible for the added protection observed with the combination of DDMS and IPC. The mechanism responsible for the nearly 90% infarct size reduction is not known and is currently under investigation.

In conclusion, the present results indicate an important endogenous role of the CYP ω-hydroxylases and their major AA metabolite, 20-HETE, in enhancing ischemia-reperfusion injury in the canine heart. For the first time, these results also implicate the combined beneficial effects of IPC and CYP ω-hydroxylase inhibitors when administered together to produce cardioprotection. Although the mechanism(s) responsible for the protection observed following CYP ω-hydroxylase inhibition are yet to be determined, these data also suggest potential therapeutic targets for intervention in myocardial ischemia and/or reperfusion injury.

GRANTS

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REFERENCES


