Reactive oxygen species are critical mediators of coronary collateral development in a canine model

Weidong Gu, Dorothee Weihrauch, Katsuya Tanaka, John P. Tessmer, Paul S. Pagel, Judy R. Kersten, William M. Chilian, and David C. Warltier. Reactive oxygen species are critical mediators of coronary collateral development in a canine model. Am.J.Physiol. Heart Circ. Physiol. 285:H1582–H1589, 2003. First published June 19, 2003; 10.1152/ajpheart.00318.2003.—Recent evidence suggests that reactive oxygen species (ROS) promote proliferation and migration of vascular smooth muscle (VSMC) and endothelial cells (EC). We tested the hypothesis that ROS serve as crucial messengers during coronary collateral development. Dogs were subjected to brief (2 min), repetitive coronary artery occlusions (1/h, 8/day, 21 day duration) in the absence (occlusion) and in the presence of N-acetylcysteine (NAC). ROS generation was assessed with dihydroethidium fluorescence. Coronary collateral blood flow (expressed as a percentage of normal zone flow) was significantly increased (71 ± 7%) in occlusion dogs after 21 days but remained unchanged (13 ± 3%) in sham dogs. Treatment with NAC attenuated increases in collateral blood flow (28 ± 8%). Brief coronary artery occlusion and reperfusion caused ROS production (256 ± 33% of baseline values), which was abolished with NAC (104 ± 12%). Myocardial interstitial fluid produced tube formation and proliferation of VSMC and EC in occlusion but not in NAC-treated or sham dogs. The results indicate that ROS are critical for the development of the coronary collateral circulation.

CORONARY COLLATERAL DEVELOPMENT is an adaptive response to chronic occlusion of major coronary arteries (7), providing an alternative source of blood supply to ischemic myocardium. Numerous studies have demonstrated that a well-developed collateral circulation is associated with higher survival rate (28), smaller infarct size (25), less aneurysm formation (13), and better ventricular function (18) compared with absent or poor collaterals in patients with coronary artery occlusion. Previous studies demonstrate that repetitive, brief episodes of myocardial ischemia are powerful stimuli for the development of coronary collateral vessels (16). However, the precise mechanisms responsible for ischemia-induced enhancement of the coronary collateral circulation are incompletely understood.

Restoration of blood flow after ischemia results in generation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radicals. ROS are commonly considered a main contributor to cell death by lipid peroxidation, oxidative modification of proteins, and DNA (33). However, recent investigations in signal transduction demonstrate that ROS at a low concentration may also serve as an intracellular messenger to induce “defensive” or “repair” mechanisms against tissue injury. For example, ROS generation during ischemic preconditioning (4, 31) or anesthetic preconditioning (32) mediate the protection against prolonged ischemia. Therefore, ROS appear to play a dual role in the ischemic event with small amounts acting as signaling molecules and large amounts causing membrane damage.

The role of ROS in angiogenesis has recently received attention. Growth factors such as vascular endothelial growth factor (VEGF) (36), EGF (14), PDGF (26), and FGF (23) require ROS activation and downstream signaling participation to exert action. Depletion of ROS impairs cell responses to mitogenic stimulation (8). Furthermore, ROS have been demonstrated to enhance proliferation of vascular smooth muscle (VSMC) and endothelial cells (EC) (3, 30), which are primary features in the process of coronary collateralization. Therefore, we tested the hypothesis that ROS play a critical role in coronary collateral development in a canine model of repetitive coronary occlusion in vivo.

MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all experimental procedures conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and were in compliance with the institution’s policies for animal research.

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accordance with the Guide for the Care and Use of Laboratory Animals (7th ed., Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205).

General preparation. Conditioned mongrel dogs were fasted overnight. Anesthesia was induced with intravenous propofol (8 mg/kg). After tracheal intubation, anesthesia was maintained with sevoflurane (3%) in 100% oxygen using positive pressure ventilation. A thoracotomy was performed under sterile conditions in the fifth intercostal space, and animals were instrumented as previously described (16). Briefly, heparin-filled catheters were secured in the descending thoracic aorta and the left and right atrium for measurement of aortic blood pressure, administration of radioactive microspheres, and fluid administration, respectively. A segment (1.5 cm) of the left anterior descending coronary artery was isolated, and a hydraulic occluder was placed around this vessel for production of repetitive coronary artery occlusions and reperusions. A Doppler flow transducer was placed immediately proximal to the LAD occluder for measurement of blood flow velocity (11). Peak reactive hyperemic response was measured as the peak blood flow velocity shift occurring immediately after LAD occlusion and reperfusion. Ischemic intensity was indicated by flow debt repayment calculated from the postocclusive reactive hyperemic response (flow debt repayment = 100 × excess reactive hyperemic flow area under curve/resting flow velocity × occlusion duration).

A perforated polyurethane catheter was implanted into the midmyocardium of the LAD perfusion territory for sampling of myocardial interstitial fluid (MIF) as previously described (34). The chest was closed in layers, and the pneumothorax was evacuated with the use of a chest tube. Each dog received antibiotics [cefazolin (4 mg/kg) and gentamicin (4.5 mg/kg)] and analgesics [epidural morphine (0.1 mg/kg) and fentanyl (5 μg/kg)]. Dogs were permitted to recover 7 to 10 days before the initiation of repetitive LAD occlusions.

Determination of myocardial blood flow. Carbonized plastic microspheres [15 ± 2 μm (± SD) in diameter] labeled with 141Ce, 51Cr, 103Ru, or 95Nb were used to measure regional myocardial perfusion as previously described (17). Approximately 2–3 × 106 microspheres were injected into the left atrium over a period of 10 s after complete occlusion of LAD. A few seconds before the microsphere injection, a timed sample of MIF was biopsied from the left atrium and harvested for measurement of collateral blood flow (transmural collateral blood flow ≥ 0.20 ml/min-1·g-1 on day 1) were excluded from analysis. Samples of MIF were collected from the intramyocardial catheter each morning before the first daily occlusion (34, 37). Four milliliters of isotonic saline were flushed into the catheter as 4 ml of aspirate were withdrawn. Samples were filtered and stored in vials containing protease inhibitor cocktail tablets (Complete Mini, EDTA free; Hoffman-La Roche, Nutley, NJ).

Detection of ROS. ROS production was detected by using the cell-permeable dye dihydroethidium (DHE) as previously described (9, 24). DHE is rapidly oxidized by superoxide to yield fluorescent ethidium. Ethidium intercalates into DNA, further amplifying the observed fluorescence. Thus an increase in DHE fluorescence in nuclei indicates generation of intracellular superoxide. Myocardium from the LAD perfusion territory was biopsied before baseline and at 0, 20 s, 40 s, 2 min, 4 min, and 6 min after a 2-min LAD occlusion and reperfusion in two additional groups of anesthetized and acutely instrumented dogs in the absence (n = 3) and presence (n = 3) of NAC (150 mg/kg iv, infused before occlusion). Myocardial samples (1.6-mm thick) were harvested by using a core biopsy needle (BARD Endoscopic Technologies; Billerica, MA). Samples were washed with PBS and immediately incubated in 5 μM DHE for 30 min. Images from myocardium harvested during each intervention were obtained by using a laser fluorescence scanning confocal microscopic imaging system (Osydsey; Noran Instruments, Madison, WI) mounted on a microscope (Optiphot; Nikon, Tokyo, Japan) and stored for off-line analysis on a computer workstation equipped with image analysis software (MetaMorph; Universal Imaging, Downingtown, PA). Excitation was produced by using a krypton-argon laser at a wavelength of 488 nm, and emitted fluorescence was measured at 610 nm. The pixel intensity of each myocyte nucleus was determined after compensating for background fluorescence by subtraction of an area without cells or minimal cytosolic fluorescence. For each experiment, five images were obtained at each intervention, and the intensity of 10 myocardial nuclei was analyzed in each image.

Cell culture of VSMC and EC. Porcine pulmonary arterial VSMC and human aortic EC were plated in plastic culture flasks with DMEM containing 10% FBS or Ham’s F-12 medium with 20% medium, respectively. The cells were maintained in a humidified atmosphere at 37°C in the presence of 5% CO2-20% O2-75% N2. All cell culture products were purchased through Invitrogen Life Technologies (Carlsbad, CA).

Cell proliferation to MIF. Mitogenic activity was assessed by testing the proliferative response of the cultured cells to MIF as previously described (37). The cells were seeded in a density of 10,000 cells/well in DMEM containing 5% FBS overnight. Cells were grown arrested for 72 h and then incubated with a 10% dilution of MIF in 0.1% (VSMC) or 0.5% FBS in DMEM (EC) for 72 h. Other cultures were treated with 0.1 and 0.5% FBS for VSMC and EC or 20% FBS for both cell types as negative and positive controls, respectively.

In vitro angiogenesis assay of MIF. Vasculogenicity was assessed by the ability of MIF to induce tube formation of EC seeded on a fibrin gel as previously described (22). Treatment with 50 ng of recombinant VEGF and unstimulated EC served as positive and negative controls, respectively.
Table 1. Hemodynamics

<table>
<thead>
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<th>Experimental Day</th>
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<tr>
<td></td>
<td>Day 1</td>
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<tr>
<td>HR, beats/min</td>
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<tr>
<td>Occlusion</td>
<td>103 ± 8</td>
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<tr>
<td>Occlusion + NAC</td>
<td>100 ± 8</td>
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<tr>
<td>Sham</td>
<td>87 ± 10</td>
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<td>MBP, mmHg</td>
<td></td>
</tr>
<tr>
<td>Occlusion</td>
<td>105 ± 5</td>
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<tr>
<td>Occlusion + NAC</td>
<td>99 ± 4</td>
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<tr>
<td>Sham</td>
<td>97 ± 4</td>
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<tr>
<td>RPP, mmHg-beats/min</td>
<td></td>
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<tr>
<td>Occlusion</td>
<td>13.8 ± 1.1</td>
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<tr>
<td>Occlusion + NAC</td>
<td>12.8 ± 1.2</td>
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<tr>
<td>Sham</td>
<td>11.1 ± 2.1</td>
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Values are means ± SE. HR, heart rate; MBP, mean aortic blood pressure; RPP, rate pressure product. *Significantly (P < 0.05) different from day 1.

Table 2. Coronary collateral blood flow to the ischemic zone

<table>
<thead>
<tr>
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<th>Experimental Day</th>
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<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Subepicardium</td>
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<tr>
<td>Occlusion</td>
<td>0.14 ± 0.02</td>
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<tr>
<td>Occlusion + NAC</td>
<td>0.17 ± 0.05</td>
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<tr>
<td>Sham</td>
<td>0.22 ± 0.09</td>
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<tr>
<td>Midmyocardium</td>
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<tr>
<td>Occlusion</td>
<td>0.09 ± 0.03</td>
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<tr>
<td>Occlusion + NAC</td>
<td>0.10 ± 0.03</td>
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<tr>
<td>Sham</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>Subendocardium</td>
<td></td>
</tr>
<tr>
<td>Occlusion</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>Occlusion + NAC</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Sham</td>
<td>0.07 ± 0.04</td>
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<tr>
<td>Transmural</td>
<td></td>
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<tr>
<td>Occlusion</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Occlusion + NAC</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Sham</td>
<td>0.11 ± 0.04</td>
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</table>

Values are means ± SE in ml·min⁻¹·g⁻¹. *Significantly (P < 0.05) different from day 1; †significantly (P < 0.05) different from the respective value in the occlusion group.
changed in NAC-treated dogs (116 ± 7, 126 ± 16, 120 ± 9, and 103 ± 10% on days 1, 7, 14, and 21, respectively) and was significantly greater than in occlusion dogs on days 14 and 21 (118 ± 11, 83 ± 17, 38 ± 12, and 11 ± 6% on days 1, 7, 14, and 21, respectively).

ROS production. ROS production (DHE fluorescence) in myocardial nuclei was significantly increased after brief coronary artery occlusion and reperfusion in untreated dogs compared with baseline measurements. In contrast, treatment with NAC completely abolished increases in ROS in response to myocardial ischemia and reperfusion (Fig. 4).

Proliferation of VSMC and EC to MIF. MIF-induced VSMC proliferation was significantly higher in occlusion dogs (33 ± 6, 63 ± 7, 88 ± 7, and 84 ± 7% increase in cell number on days 1, 7, 14, and 21, respectively) compared with the negative control (0.1% FBS). In contrast, mitogenic activity of MIF on VSMC proliferation (Fig. 5) was absent in the occlusion + NAC and the sham groups. Similarly, MIF obtained from occlusion dogs induced EC proliferation (63 ± 8, 51 ± 7, 38 ± 6, and 47 ± 8% increase in cell number on days 1, 7, 14, and 21, respectively). EC proliferation was not induced by MIF from NAC-treated or sham dogs (Fig. 6).

Expression of VEGF in MIF. VEGF protein in MIF was increased on day 3 and reduced thereafter in

**Fig. 2.** A: representative reactive hyperemic response from an occlusion dog on days 1, 7, 14, and 21. Peak reactive hyperemic response progressively decreased after repetitive left anterior descending coronary artery (LAD) occlusions. B: representative reactive hyperemic response from an occlusion + NAC dog. Peak reactive hyperemic response was higher in NAC dogs compared with occlusion dogs on days 14 and 21. C: representative reactive hyperemic response from a sham dog. Peak reactive hyperemic response remained unchanged over the time course of experiment. D: peak reactive hyperemia in occlusion, NAC-treated, and sham dogs. Data are means ± SE. *Significantly (P < 0.05) different from day 1; †significantly (P < 0.05) different from the respective value in the occlusion group.

**Fig. 3.** Flow debt repayment in occlusion and occlusion + NAC dogs. Flow debt repayment remained unchanged over 21 days in NAC-treated dogs and was greater than in occlusion dogs on days 14 and 21. Data are means ± SE. *Significantly (P < 0.05) different from the respective value in the occlusion group.

**Fig. 4.** Histograms depicting dihydroethidium fluorescence intensity before and after a 2-min LAD occlusion and reperfusion in the absence (occlusion) or presence of pretreatment with NAC (occlusion + NAC). Fluorescence intensity was increased in occlusion dogs but not in NAC-treated dogs. *Significantly (P < 0.05) different from baseline; †significantly (P < 0.05) different from the respective value in the occlusion group.
occlusion dogs. In NAC-treated dogs, VEGF expression remained at high levels throughout the 3 wk of repetitive occlusions (Fig. 8).

**DISCUSSION**

The present investigation provides several new insights into coronary collateral development. The key findings include 1) in contrast to the enhancement of the coronary collateral circulation in occlusion dogs, the antioxidant NAC attenuated increases in collateral blood flow and decreases in PRH in response to repetitive LAD occlusions; 2) brief coronary artery occlusion produced increases in ROS production on reperfusion, whereas treatment with NAC inhibited ROS generation; 3) vasculogenic properties of MIF, assessed by in vitro angiogenesis assays, were increased in occlusion dogs but absent in NAC-treated and sham dogs. Similarly, MIF-induced proliferation of VSMC and EC were reduced in the occlusion/NAC and sham groups compared with the occlusion groups; 4) VEGF expression was increased early (3 days) after the initiation of repetitive LAD occlusions but waned thereafter. Treatment with NAC augmented VEGF expression throughout the duration of the experiment. Thus the present results demonstrate that depletion of ROS abolishes coronary collateral development. This occurs concomitant with reduced mitogenic activity of MIF in the region exposed to repetitive brief ischemic episodes. Taken together, these data indicate that ROS are critical for the development of the coronary collateral circulation.

Coronary angiogenesis is a highly regulated and complex cascade of events that requires degradation of surrounding extracellular matrix, and proliferation and migration of VSMC and EC. ROS have recently been implicated as necessary components of angiogenic signaling in vitro (5). For example, low concentrations of hydrogen peroxide (0.1–10 μM) stimulate proliferation and tube formation of bovine aortic EC through induction of the transcription factor Ets-1 (38). Ets-1 regulates the expression of genes involved in extracellular matrix degradation, including urokinase plasminogen activator (uPA) and matrix metalloproteinase (MMP) genes. VEGF, a major regulator of angiogenesis, is known to induce the expression of these genes through the activation of the extracellular signal-regulated kinase (ERK) pathway (39). Thus, the observed decrease in VEGF expression in NAC-treated dogs suggests that ROS are essential for the enhancement of angiogenic activity in occlusion dogs.
minogen activator and matrix metalloproteinase-1. Intracellular ROS production and NF-κB activation have been shown to mediate VEGF-induced VSMC migration and proliferation (36). Our results provide the first evidence to indicate that ROS mediate coronary collateral growth in response to repetitive myocardial ischemic stimuli in vivo. Collateral blood flow to the ischemic zone was 28% of normal zone flow after 3 wk of repetitive occlusions in NAC-treated dogs compared with 71% in occlusion dogs. The results are in agreement with the recent in vitro investigation by Lelkes et al. (20), who demonstrated that exposure of human microvascular EC to brief hypoxia and reoxygenation increases ROS generation and stimulates tube formation. ROS scavengers, including pyrrolidine dithiocarbamate, superoxide dismutase, and catalase, blocked tube formation.

Although there is mounting evidence that brief myocardial ischemia increases ROS production after reperfusion, the in vivo evidence is limited. In an isolated perfused rat heart model, Henry et al. (12) demonstrated that ROS production was dramatically increased after reperfusion in myocardium subjected to brief ischemia (5 min). With the use of the DHE fluorescence technique, the present results provide direct evidence that 2 min of ischemia causes an increase in ROS production in myocardium immediately after reperfusion. NAC is a thiol-containing, low-molecular-weight compound that scavenges both extracellular and intracellular ROS (2). It has been demonstrated that infusion of NAC increases endogenous concentrations of glutathione and decreases protein carbonyl concentrations (a marker of protein oxidation) in rats treated with Fe²⁺/H₂O₂ (29). Alberola et al. (1) reported that depletion of myocardial glutathione during myocardial ischemia and reperfusion was not observed in dogs treated with NAC (150 mg/kg iv). In agreement with these investigations, the present results demonstrate that 150 mg/kg NAC inhibits the increase in ROS production during reperfusion and suggests that the dose of NAC used was capable of exerting antioxidant effects in vivo.

The present experiments conducted in vitro characterize the mitogenic and vasculogenic activity of MIF, as assessed by proliferation of VSMC and EC and tube formation of EC, respectively. Cell proliferation and tube formation were not induced by MIF from NAC-treated dogs compared with MIF harvested from occlusion dogs. This was true despite an increase in VEGF in the MIF from NAC-treated dogs. These results suggest that ROS are needed in growth factor signaling to produce cell proliferation and tube formation. Most likely, NAC in MIF (sampled after the infusion of NAC) inhibited growth factor-induced ROS generation and thereby decreased the mitogenic and vasculogenic activity of MIF. This concept is supported by recent evidence demonstrating that ROS are downstream mediators of VEGF signaling. VEGF receptor activation causes a rapid increase in the intracellular generation of hydrogen peroxide, and inhibition of ROS production by antioxidants attenuates early VEGF signaling (5).

Our previous study (21) demonstrates that VEGF expression in MIF peaked at day 3 and waned thereafter in dogs subjected to repetitive coronary artery occlusions. The present results confirm our previous findings and further demonstrate that VEGF expression is elevated for 21 days in dogs treated with NAC. It has been shown that myocardial ischemia upregulates the expression of VEGF in the canine heart (15). The present results are in agreement that VEGF expression increased early after the onset of repetitive coronary artery occlusions in occlusion dogs. The decrease of VEGF expression after day 7 occurred concomitant with coronary collateral development, increases in collateral perfusion, and a reduction in the severity of myocardial ischemia. Treatment with NAC inhibited coronary collateral development in the ischemic region. The maintenance of PRH and flow debt repayment at a high level throughout the experiment suggested that the intensity of myocardial ischemia was not substantially reduced in NAC-treated dogs, and this may have induced continuous stimulation and sustained expression of VEGF in MIF over the 3-wk time course of the experiments. In addition to VEGF, coronary collateral development is regulated by other growth factors, such as EGF, PDGF, and FGF (23). Our present findings that MIF still possessed angiogenic properties in the presence of low concentrations of VEGF after 1 wk in occlusion dogs and our previous findings that basic FGF plays a role later (days 12–14) during collateral development (37) indicate that multiple cytokines contribute to collateral development. PDGF, FGF-2, and transforming growth factor-β1 rapidly induce the generation of intracellular superoxide (35), indicating that ROS serve as signaling molecules in response to these cytokines as well.

In the present investigation, NAC significantly attenuated but did not completely abolish the development of coronary collaterals. We have previously demonstrated (21) that the nitric oxide synthase inhibitor, N⁵-nitro-L-arginine methyl ester, inhibits collateral development in response to myocardial ischemia. Interestingly, a nitric oxide-ROS pathway has been implicated during ischemia-induced signal transduction in late preconditioning (6). Nitric oxide and ROS, both produced during brief ischemia, are thought to react and generate peroxynitrite and hydroxyl radical and further activate several transcription factors including NF-κB and activator protein-1 (AP-1). Activation of NF-κB and AP-1 has previously been shown to increase the expression of proteins such as matrix metalloproteinases (10) and monocyte chemotactic protein-1 (19), which are induced during angiogenesis. Our earlier work (21) demonstrating that nitric oxide is required and our present findings that ROS are critical mediators of coronary collateral development suggest that the nitric oxide-ROS pathway may also be involved in coronary collateralization. This hypothesis will require further investigation.

The present results should be interpreted within the constraints of several potential limitations. First, the half-life of NAC is 5.58 h (27). Therefore, it is possible...
that NAC was less effective in eliminating ROS during the seventh and eighth occlusion of each day. Second, rate pressure produce (RPP) was decreased on days 14 and 21 compared with day 1 in the NAC group. Decreases in the hemodynamic determinants of myocardial oxygen consumption may have reduced the intensity of ischemic stimuli during repetitive coronary artery occlusion in NAC-treated dogs. Although it is possible that a decrease in ischemic intensity could account for impaired collateral development in NAC-treated dogs, maintenance of flow debt repayment over the time course of the experiment indicates that coronary artery occlusion produced similar degrees of myocardial ischemia on day 1 compared with day 21. Higher flow debt repayment in the NAC dogs also suggests that ischemic stimulus was greater in NAC dogs than that in occlusion dogs on days 14 and 21. In addition, coronary collateral blood flow was significantly less in the NAC group compared with the occlusion group on day 7 despite similar RPP in both groups. Therefore, it is unlikely that differences in collateral blood flow between the occlusion and occlusion + NAC groups were related solely to differences in hemodynamics or intensity of ischemic stimuli.

In conclusion, the present results demonstrate that coronary collateral development and in vitro mitogenic and vascuogenic activity of MIF are increased in dogs subjected to repetitive coronary artery occlusions. This adaptive response to coronary artery occlusion is impaired by the antioxidant NAC. The findings indicate that ROS production serves as a critical event to induce development of the coronary collateral circulation in response to repetitive LAD occlusions in vivo.

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DISCLOSURES

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REFERENCES


