Endothelium-Derived Hyperpolarizing Factor Synthase (Cytochrome P450 2C9) Is a Functionally Significant Source of Reactive Oxygen Species in Coronary Arteries

Ingrid Fleming, U. Ruth Michaelis, Daniel Bredenkötter, Beate Fisslthaler, Faramarz Dehghani, Ralf P. Brandes, Rudi Busse

Abstract—In the porcine coronary artery, a cytochrome P450 (CYP) isozyme homologous to CYP 2C8/9 has been identified as an endothelium-derived hyperpolarizing factor (EDHF) synthase. As some CYP enzymes are reported to generate reactive oxygen species (ROS), we hypothesized that the coronary EDHF synthase may modulate vascular homeostasis by the simultaneous production of ROS and epoxyeicosatrienoic acids. In bradykinin-stimulated coronary arteries, antisense oligonucleotides against CYP 2C almost abolished EDHF-mediated responses but potentiated nitric oxide (NO)-mediated relaxation. The selective CYP 2C9 inhibitor sulfaphenazole and the superoxide anion (O$_2^-$) scavengers Tiron and nordihydroguaretic acid also induced a leftward shift in the NO-mediated concentration-relaxation curve to bradykinin. CYP activity and O$_2^-$ production, determined in microsomes prepared from cells overexpressing CYP 2C9, were almost completely inhibited by sulfaphenazole. Sulfaphenazole did not alter the activity of either CYP 2C8, the leukocyte NADPH oxidase, or xanthine oxidase. ROS generation in coronary artery rings, visualized using either ethidium or dichlorofluorescein fluorescence, was detected under basal conditions. The endothelial signal was attenuated by CYP 2C antisense treatment as well as by sulfaphenazole. In isolated coronary endothelial cells, bradykinin elicited a sulfaphenazole-sensitive increase in ROS production. Although 11,12 epoxyeicosatrienoic acid attenuated the activity of nuclear factor-κB in cultured human endothelial cells, nuclear factor-κB activity was enhanced after the induction or overexpression of CYP 2C9, as was the expression of vascular cell adhesion molecule-1. These results suggest that a CYP isozyme homologous to CYP 2C9 is a physiologically relevant generator of ROS in coronary endothelial cells and modulates both vascular tone and homeostasis. (Circ Res. 2001;88:44-51.)

Key Words: coronary artery ▪ cytochrome P450 ▪ endothelium-derived hyperpolarizing factor ▪ NADPH oxidase ▪ reactive oxygen species

The bioavailability of nitric oxide (NO) within the vascular wall and, as a consequence, NO-mediated relaxation is attenuated by an elevation in superoxide anion (O$_2^-$) production. Enzymes capable of generating reactive oxygen species (ROS) within the vasculature are, for example, NO synthases (NOS), cyclooxygenases, lipoxygenases, xanthine oxidase, and NADPH oxidase, all of which are reported to be functional in endothelial cells. Diphenyleneiodonium has been used extensively to characterize ROS-generating enzymatic systems; however, this compound inactivates flavoproteins during electron-transfer reactions and completely inhibits the activity of all isoforms of NOS, xanthine oxidase, and NADPH oxidase. Thus, although pharmacological studies using isolated arteries have demonstrated that the production of ROS is markedly increased in animal models of hypertension, atherosclerosis, and heart failure, the relative contribution of each of the potential O$_2^-$-producing enzymes to the overall ROS production remains to be elucidated.

In the coronary system, NO inhibits the activity of the cytochrome P450 (CYP)-like endothelium-derived hyperpolarizing factor (EDHF) synthase. A decrease in the bioavailability of NO, as demonstrated in various states associated with endothelial dysfunction, alleviates this intrinsic inhibition so that the activity of the EDHF synthase and the production of vasodilator epoxyeicosatrienoic acids (EETs) are increased. As a consequence of this interaction, vascular responsiveness is thought to be at least partially maintained despite the apparent loss of NO.

Interest in the consequences of vascular CYP expression has focused on the vascular effects of EET production, and little attention has been paid to the fact that O$_2^-$, hydrogen...
peroxide, and hydroxyl radicals can also be generated during the CYP reaction cycle when the electrons for the reduction of the central heme iron are transferred on the activated bound oxygen molecule.11-13 Therefore, it is conceivable that CYP epoxygenases, which have recently been detected in coronary endothelial cells,14-17 may contribute to the generation of oxygen-derived free radicals within the vascular wall. The aim of the present investigation was to determine whether the putative coronary EDHF synthase CYP 2C9 is a physiologically relevant source of ROS.

Materials and Methods

Materials

OxyBURST green H2FF BSA (oxygenBURST) and L’,7’,3’,4’ dichlorodihydrofluorescein diacetate (H2DCF-DA) were from Molecular Probes, DMEM-F12 and M-199 medium from Gibco, and dibenzylfluorescein from NatuTec. Diphenylethenediomium was from Alexis, and 11,12-EET was purchased from Cayman Chemical. All other chemicals were obtained from Sigma.

Preparation and Transfection of Porcine Coronary Arteries

Porcine epicardial artery segments (~40 mm length; mean external diameter 2.4 to 2.8 mm) were excised, side branches were sealed with surgical clips, and the segment was cannulated at both ends and placed into vessel chambers. After equilibration, MEM containing 10% FCS, 2.5 μg/mL FITC-labeled antisense or sense oligonucleotides, and 20 μM transfection reagent (Superfect, Qiagen) were applied luminally and left in contact for 3 hours under a maintained transmural pressure of 90 mm Hg. Thereafter, coronary arteries were perfused (5 mL/h, 37°C) with MEM containing 2% FCS for 18 to 20 hours. After incubation, segments were cut into rings for organ chamber studies and precontracted with U46619 (0.1 to 1 μM) and placed on a glass slide. Hydroethidine (10 μM) was applied topically to each section before sealing with a coverslip as described.21 After a 30-minute incubation period, during which hydroethidine was oxidized to the fluorophore ethidium, images were obtained using an imaging system (λ Ex: 520, Em: 605 nm; Attofluor).

Transfection of Cultured Endothelial Cells

Porcine coronary artery endothelial cells were prepared as described elsewhere,22 and human umbilical vein endothelial cells for transfection were purchased from Cell Systems/Clonetics. Subconfluent cells (~80% confluent) were exposed for 4 hours to 2.63 μg/mL plasmid DNA (pcDNA3.1) containing the coding region of CYP 2C9 under the control of a cytomegalovirus promoter. Thereafter, cells were maintained in medium containing 4% FCS for an additional 48 hours. The expression of CYP 2C9 and activity of nuclear factor-κB (NF-κB) were assessed as described.16,23

Immunofluorescence Experiments

Coronary artery segments were fixed with formaldehyde (2% in PBS). After extensive washing, the segments were permeabilized with Triton X-100 (0.2%) and incubated in glycine (100 mmol/L) for 10 minutes. After extensive washing, segments were coincubated with a specific polyclonal CYP 2C antibody (kindly provided by Dr E. Morgan, Atlanta, Ga) and a monoclonal actin antibody (Sigma) for 2 hours followed by fluorescein-conjugated and Texas Red-conjugated secondary antibodies (Dianova) for 60 minutes. Human endothelial cells were fixed in formaldehyde, permeabilized, and coincubated with a CYP 2C antibody and a monoclonal vascular cell adhesion molecule-1 (VCAM-1) antibody (R&D Systems). After incubation with fluorescein and Texas Red–coupled secondary antibodies, the preparations were mounted and viewed using a confocal microscope.

Statistics

Data are expressed as mean ± SEM, and statistical evaluation was performed using Student’s t test for paired or unpaired data, one-way ANOVA followed by a Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of P < 0.05 were considered statistically significant. pD2 (−log EC50) values were calculated by nonlinear regression of the concentration-relaxation curves to bradykinin.

Results

Effects of CYP 2C Downregulation and Inhibition on NO-Mediated Relaxation

Antisense oligonucleotides derived from the cDNA sequences of human CYP 2C8 and 2C9, which have previously been shown to attenuate CYP 2C protein expression and EDHF-mediated responses,15,20 were used to downregulate CYP 2C expression.

Treatment of coronary arteries with the transfection reagent alone or sense or scrambled oligonucleotides failed to affect NO-mediated relaxation, as described previously.15 However, antisense oligonucleotide treatment, which decreased CYP expression (see Figures 4C and 4D), induced a
marked leftward shift in the concentration-relaxation curve to bradykinin (Figure 1A). Although bradykinin-induced relaxation was detectable in rings maintained for up to 60 hours, EDHF-mediated relaxation decreased in a time-dependent manner. On the other hand, NO-mediated relaxation increased, inducing a leftward shift in the relaxation-response curve, accounting for the slight difference in the relaxation of solvent-treated rings in Figures 1A and 1B.

In freshly isolated arteries, the selective CYP 2C9 inhibitor sulfaphenazole induced a pronounced leftward shift in the concentration-relaxation curve to bradykinin (Figure 1B; pD₂ values being 7.87±0.06 and 8.54±0.03 in the presence of solvent and sulfaphenazole, respectively, P<0.001, n=12). An improvement in the bradykinin-induced NO-mediated relaxation of coronary artery rings was also observed in the presence of the O₂⁻ scavenger Tiron or the dual CYP and lipoxygenase inhibitor nordihydroguaretic acid (pD₂ values being 8.26±0.09, 8.70±0.08, and 8.77±0.08 in the presence of solvent, Tiron, and nordihydroguaretic acid, respectively, P<0.001, n=8). None of the antioxidants investigated exerted a significant effect on the contraction to U46619.

Effect of Sulfaphenazole on ROS Production and Substrate Conversion by CYP 2C8 and 2C9
To determine whether the putative coronary EDHF synthase generates ROS, supersomes isolated from cells overexpressing either CYP 2C8 or 2C9 were incubated with the oxidative-sensitive fluorogenic reagent oxyBURST. ROS generation was observed using both CYP 2C8- and 2C9-containing supersomes. The generation of ROS by both CYP enzymes was attenuated by 17-ODYA (by 36±4% and 63±4%, respectively) and miconazole (by 26±7% and 87±3%, respectively). Sulfaphenazole selectively inhibited ROS generation by CYP 2C9 (Figure 2A). The IC₅₀ value for sulfaphenazole on ROS generation by CYP 2C9–containing microsomes was 1.72±0.35 μmol/L, whereas 100 μmol/L sulfaphenazole inhibited CYP 2C8–derived ROS generation by only 70%.

To determine the selectivity of sulfaphenazole for CYP 2C9, CYP activity was assessed as the dealkylation of the CYP 2C8/9 substrate DBF to fluorescein over 30 minutes in the absence and presence of sulfaphenazole (Sulfa, 10 μmol/L) and DPI (10 μmol/L). Results are mean±SEM of 3 separate experiments, each performed in quadruplicate. **P<0.01; ***P<0.001.

Effect of sulfaphenazole on ROS generation by NADPH oxidase, xanthine oxidase, and CYP 2C9. ROS formation in PMA-stimulated human leukocytes containing NADPH oxidase, a purified preparation of xanthine oxidase (0.1 μU/mL) and xanthine (100 μmol/L), and CYP 2C9 supersomes was measured using compound 5–enhanced chemiluminescence. Experiments were performed in the absence (CTL) and presence of sulfaphenazole (Sulfa, 10 μmol/L) and DPI (10 μmol/L). Results are mean±SEM of 3 separate experiments, each performed in quadruplicate. **P<0.01.
Sulfaphenazole markedly attenuated the activity of CYP 2C9 but was without effect on CYP 2C8 (Figure 2B) or CYP 3A4 (data not shown).

Sensitivity of Superoxide-Generating Enzymes to Sulfaphenazole

To ensure that sulfaphenazole did not affect ROS generation by the NADPH oxidase or xanthine oxidase, the generation of ROS by human leukocytes and a purified preparation of xanthine oxidase was assessed using compound 5–enhanced chemiluminescence and compared with that of CYP 2C9–containing supersomes. In isolated human leukocytes, neither bradykinin (100 nmol/L) nor 11,12-EET (up to 10 μmol/L) was able to activate the NADPH oxidase (data not shown), but phorbol 12-myristate 13-acetate (PMA, 1 μmol/L) induced a significant (20-fold) increase in ROS production. Sulfaphenazole failed to affect ROS generation under either basal conditions (data not shown) or after stimulation with PMA (Figure 3). Similarly, sulfaphenazole was without effect on ROS production by xanthine oxidase, which was abolished by oxypurinol (100 μmol/L, 95.7±2.0% inhibition, n=3), but inhibited CYP 2C9–induced compound 5–chemiluminescence (Figure 3). ROS generation by PMA-stimulated leukocytes, xanthine oxidase, and CYP 2C9 was completely abolished by DPI or the addition of superoxide dismutase (100 U/mL).

Generation of ROS in Porcine Coronary Endothelial Cells

In sections of porcine coronary artery stained with dihydroethidine, a fluorescent signal was detected in both endothelial and smooth muscle cells. However, after treatment of arterial segments with CYP 2C antisense oligonucleotides, CYP 2C protein levels were decreased and the fluorescent ethidium signal in the endothelium was markedly attenuated (Figures 4A through 4D). CYP 2C antisense oligonucleotides did not affect the fluorescent signal in the vascular smooth muscle. Incubation of arterial rings with sulfaphenazole also selectively attenuated the ethidium fluorescent signal detected in endothelial cells without affecting the signal in either the media or adventitia (Figures 4E and 4F).

To determine whether the endothelial agonist bradykinin, which elicits EDHF-mediated responses, elevated the produc-

**Figure 4.** In situ detection of ROS production in the porcine coronary artery. A and B, Fluorescent photomicrographs obtained at identical settings of sections of the same porcine coronary artery labeled with the oxidative dye hydroethidine 18 hours after treatment with sense (A) and antisense (B) CYP 2C oligonucleotides. Inset shows an overview of the signal obtained in a separate experiment. C and D, Immunohistochemical staining of CYP 2C (green) and actin (red) in sections of the same porcine coronary artery 18 hours after treatment with sense (C) and antisense (D) CYP 2C oligonucleotides. Bar=100 μm. E and F, Fluorescent photomicrographs of sections of a porcine coronary artery labeled with hydroethidine after incubation with solvent (E) and sulfaphenazole (10 μmol/L) (F). Inset shows an overview of the signal obtained in a separate experiment, and results presented are representative of data obtained in 6 separate experiments.

**Figure 5.** Time course of ROS generation by bradykinin-stimulated porcine coronary artery endothelial cells. H$_2$DCF–DA–loaded endothelial cells were pretreated with N-nitro-L-arginine (300 μmol/L) and stimulated with bradykinin (100 nmol/L) in the absence (CTL) and presence of sulfaphenazole (Sulfa, 10 μmol/L) and DCF fluorescence monitored over 10 minutes using a confocal microscope. A, DCF fluorescence in unstimulated cells (CTL) and in cells incubated with sulfaphenazole (Sulfa, 10 μmol/L). Bar=100 μm. B, Time course of the bradykinin (100 nmol/L)-induced increase in DCF fluorescence in the absence and presence of sulfaphenazole (Sulfa, 10 μmol/L). Results are presented as the mean±SEM of 3 separate experiments.
Effect of CYP Induction and CYP 2C9 Overexpression on NF-κB and VCAM-1

11,12-EET, which is the major epoxygenase product detectable in β-naphthoflavone–stimulated porcine coronary artery endothelial cells, exerts anti-inflammatory effects on tumor necrosis factor-α (TNF-α)–stimulated endothelial cells by preventing the activation of NF-κB. Therefore, we determined the effects of CYP induction and CYP 2C9 overexpression on the activity of NF-κB.

In confluent primary cultures of human endothelial cells, 11,12-EET had a biphasic effect on NF-κB, consisting of a transient increase followed by a decrease in DNA binding. Pretreatment of endothelial cells with 11,12-EET attenuated the activation of NF-κB by TNF-α (Figure 6A). Enhancing CYP expression by incubating endothelial cells with the Ca2+ antagonist nifedipine (0.1 μmol/L, 18 hours) increased both the basal and TNF-α–induced increase in NF-κB DNA binding (Figure 6B). Overexpression of CYP 2C9 increased endothelial generation of 11,12-EET by 453±40% (n=4) and ROS production by 283±22% (n=3) compared with nontransfected endothelial cells and markedly enhanced the binding of DNA by NF-κB (Figure 6C). The addition of anti-p65 antibody to the assay caused a supershift in the gel mobility of the NF-κB protein-DNA complex (Figure 6C).

The expression of VCAM-1 is controlled by NF-κB and is reported to be increased by ROS but decreased by 11,12-EET. To determine which of these influences predominates in CYP-expressing cells, we assessed the expression of VCAM-1 in human endothelial cells transfected with CYP 2C9. VCAM-1 was not detected in unstimulated endothelial cells but was clearly evident 10 hours after transfection of endothelial cells with CYP 2C9, an effect not observed in transfected cells treated with sulfaphenazole (Figure 7). There seemed to be no paracrine effect of CYP products on VCAM-1 expression in neighboring endothelial cells, because the only cells that stained positively for VCAM-1 were those expressing CYP 2C9.

Stimulation of CYP 2C9–transfected endothelial cells with TNF-α did not affect the level of CYP 2C9 protein but markedly increased (~14-fold) the expression of VCAM-1 in the entire cell population. However, VCAM-1 expression was greater in cells overexpressing CYP 2C9, and the inclusion of sulfaphenazole reduced VCAM-1 expression to the level of TNF-α–stimulated cells transfected with the empty vector (Figure 7).

In native porcine coronary endothelial cells, which express CYP 2C9 and stain ethidium positive, VCAM-1 was also detected (Figure 8). Treatment with CYP 2C9 antisense oligonucleotides markedly attenuated the basal expression of VCAM-1 and exerted a moderate effect on the expression of VCAM-1 after stimulation of segments with TNF-α (Figure 8).

Discussion

CYP 2C9 has previously been reported to generate 11,12-EET in coronary endothelial cells and plays a crucial role in EDHF-mediated hyperpolarization and relaxation. In the present study, we have demonstrated that, in both cultured and native porcine coronary endothelial cells, CYP 2C9 is also a physiologically relevant source of ROS. Overexpres-
sion of CYP 2C9 in coronary artery endothelial cells markedly increases 11,12- and 8,9-EET generation as well as that of ROS. The consequences of superoxide anion or hydrogen peroxide production by CYP 2C and VCAM-1 antibodies (A) or subjected to Western blotting (B). Because TNF-α induced a marked increase in VCAM-1 expression (~16-fold), the blots shown were exposed for different times to demonstrate the modulatory effect of CYP 2C9 expression in the absence and presence of sulfaphenazole (Sulfa, 10 μmol/L). Similar results were obtained using 3 different cell preparations. Bar=50 μm.

Figure 7. Effects of CYP 2C9 transfection on the expression of VCAM-1. Cultured human endothelial cells were transfected with either an empty vector or with CYP 2C9 10 hours before stimulation with TNF-α (500 U/mL). After an additional 4 hours, cells were either fixed and labeled with CYP 2C and VCAM-1 antibodies (A) or subjected to Western blotting (B). Because TNF-α induced a marked increase in VCAM-1 expression (~16-fold), the blots shown were exposed for different times to demonstrate the modulatory effect of CYP 2C9 expression in the absence and presence of sulfaphenazole (Sulfa, 10 μmol/L). Similar results were obtained using 3 different cell preparations. Bar=50 μm.

Although accepted to play a role in the pathophysiology of hypertension, atherosclerosis, and heart failure, it is not generally appreciated that ROS, such as O$_2^-$ and hydrogen peroxide, are intracellular signaling molecules that are involved in the regulation of vascular tone under normal conditions. In the pulmonary artery, for example, so much O$_2^-$ is generated in response to an increase in fluid shear stress that the production of NO can only be demonstrated in the presence of high concentrations of superoxide dismutase. The most frequently studied sources of ROS in endothelial cells are the NADPH oxidase, xanthine oxidase, cyclooxygenase, and eNOS. It is only relatively recently that CYP enzymes, some of which may generate ROS, have been identified in the vasculature, both in endothelial and vascular smooth muscle cells. In the porcine coronary artery, the putative EDHF synthase may be the dominant source of endothelial O$_2^-$ in vivo, as it can be continuously activated by the rhythmic vessel distension that occurs during the cardiac cycle. Given that many constitutively expressed enzymes are able to generate ROS, it is difficult to identify the enzymatic source of vascular O$_2^-$.

Most studies have relied on pharmacological agents, such as L-arginine analogues, oxypurinol, or, in the present study, sulfaphenazole, to suggest a physiological role for ROS generation by NOS, xanthine oxidase, and CYP 2C9, respectively. The most convincing evidence obtained in support of our hypothesis that CYP 2C is a physiologically relevant source of ROS was provided using an antisense approach. Indeed, after treatment with CYP 2C antisense oligonucleotides, free radical generation in the endothelium of porcine coronary arteries was decreased and NO-mediated relaxations were significantly enhanced. Although it is not strictly correct to compare results obtained in 2 different models, it is interesting to note that the extent of the shift in the concentration-relaxation curve to an endothelial agonist in CYP 2C antisense-treated arteries was much more pronounced than that resulting from knockout of the gp91phox component of the endothelial NADPH oxidase in the mouse aorta. Homology among the different CYP 2C isoforms is exceedingly high, and using the antisense approach and antibodies used in the present study, it is not possible to differentiate between the expression of CYP 2C8 and 2C9. However, the finding that sulfaphenazole, a selective inhibitor of CYP 2C9, inhibits EDHF-mediated responses and potentiates NO-mediated relaxation in the porcine coronary artery suggests that the CYP isoform responsible for the generation of EDHF/EET and ROS is a porcine equivalent of CYP 2C9. A possible interaction between CYP 2C-derived EETs and the NADPH oxidase could also be discounted, because a CYP inhibitor-sensitive production of ROS could be demonstrated in coronary artery endothelial cells treated with CYP 2C antisense oligonucleotides.
with antisense oligonucleotides against p22phox (authors’ unpublished data, August 2000), an approach that inhibits O$_2^-$ production in vascular smooth muscle cells. Moreover, in in vitro assays, NADPH oxidase, xanthine oxidase, CYP 2C8, and CYP 3A4 were not sensitive to sulfaphenazole, leaving CYP 2C9 as the most probable candidate for the sulfaphenazole-sensitive formation of ROS in endothelial cells.

To date, when considering the consequences of vascular CYP activation, most attention has been focused on the generation of vasoactive arachidonic acid metabolites by CYP epoxygenases and $\omega$-hydroxylases. In addition to activating $\text{Ca}^{2+}$-dependent potassium channels and tyrosine kinases, 40,41 11,12-EET, for example, has been reported to exert an anti-inflammatory effect in endothelial cells by inhibiting the activation of NF-$\kappa$B and decreasing the cytokine-induced expression of VCAM-1.17 However, there is indirect evidence suggesting that another CYP-derived product exerts distinctly different effects on endothelial signaling. For example, overexpression of the EET-generating epoxygenase CYP 2J2 in bovine endothelial cells attenuated the TNF-$\alpha$-induced activation of NF-$\kappa$B but to a lesser extent than the exogenously applied EET.17 In the present study, elevating CYP 2C protein levels by incubating endothelial cells with nifedipine or by overexpressing CYP 2C9 markedly enhanced the basal activity of NF-$\kappa$B. A direct relationship between CYP expression and NF-$\kappa$B could be demonstrated in that the CYP-induced increase in NF-$\kappa$B activity could be prevented by sulfaphenazole both under basal conditions and after cell stimulation with TNF-$\alpha$. Moreover, the VCAM-1 expression elicited by CYP overexpression under basal conditions was completely abolished by sulfaphenazole, whereas the expression of VCAM-1 induced by TNF-$\alpha$ in CYP-expressing cells was only partially sensitive to the CYP inhibitor. Because NF-$\kappa$B is a redox-sensitive transcription factor, and 11,12-EET alone attenuates its activation,17 CYP-derived ROS seem to be responsible for the increase in NF-$\kappa$B activity and the subsequent induction of VCAM-1 in CYP 2C9-expressing endothelial cells.

Taken together, the results of the present study suggest that CYP 2C9, expressed in coronary endothelial cells, is not only crucial for the generation of the potent vasorelaxant 11,12-EET but is a potential major source of ROS within the coronary wall. Thus, whereas the anti-inflammatory CYP product EDHF/EET may be the dominant endothelium-derived vasoactive autacoid in states associated with a manifest endothelial dysfunction, the enhanced activation of the CYP-like EDHF synthase may eventually be detrimental to vascular homeostasis as a consequence of the simultaneous generation of EETs and ROS.

Acknowledgments

This study was supported by the Heinrich and Fritz Riese-Stiftung, Deutsche Forschungsgemeinschaft (FI 830-1/1), and the Institut de Recherches Internationales Servier. The authors are indebted to Isabel Winter and Stergiani Hauk for expert technical assistance and to Dr David Harrison (Atlanta, Ga) for helpful suggestions during the preparation of this manuscript.

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Circ Res. 2001;88:44-51
doi: 10.1161/01.RES.88.1.44

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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