Electron Spin Resonance Characterization of Vascular Xanthine and NAD(P)H Oxidase Activity in Patients With Coronary Artery Disease
Relation to Endothelium-Dependent Vasodilation

Stephan Spiekermann, MD*; Ulf Landmesser, MD*; Sergey Dikalov, PhD; Martin Bredt, MD; Graciela Gamez, BS; Helma Tatge, BS; Nina Reepschläger, BS; Burkhard Hornig, MD; Helmut Drexler, MD; David G. Harrison, MD

Background—Increased inactivation of nitric oxide by superoxide (O$_2^-$) contributes to endothelial dysfunction in patients with coronary disease (CAD). We therefore characterized the vascular activities of xanthine oxidase and NAD(P)H oxidase, 2 major O$_2^-$-producing enzyme systems, and their relationship with flow-dependent, endothelium-mediated vasodilation (FDD) in patients with CAD.

Methods and Results—Xanthine- and NAD(P)H-mediated O$_2^-$ formation was determined in coronary arteries from 10 patients with CAD and 10 controls by using electron spin resonance spectroscopy. Furthermore, activity of endothelium-bound xanthine oxidase in vivo and FDD of the radial artery were determined in 21 patients with CAD and 10 controls. FDD was measured before and after infusion of the antioxidant vitamin C (25 mg/min i.a.) to determine the portion of FDD inhibited by radicals. In coronary arteries from patients with CAD, xanthine- and NAD(P)H-mediated O$_2^-$ formation was increased compared with controls (xanthine: 12±2 versus 7±1 nmol O$_2$ /µg protein; NADH: 11±1 versus 7±1 nmol O$_2$ /µg protein; and NADPH: 12±2 versus 9±1 nmol O$_2$ /µg protein; each P<0.05). Endothelium-bound xanthine oxidase activity was increased by >200% in patients with CAD (25±4 versus 9±1 nmol O$_2$ /µL plasma per min; P<0.05) and correlated inversely with FDD (r=−0.55; P<0.05) and positively with the effect of vitamin C on FDD (r=0.54; P<0.05).

Conclusions—The present study represents the first electron spin resonance measurements of xanthine and NAD(P)H oxidase activity in human coronary arteries and supports the concept that increased activities of both enzymes contribute to increased vascular oxidant stress in patients with CAD. Furthermore, the present study suggests that increased xanthine oxidase activity contributes to endothelial dysfunction in patients with CAD and may thereby promote the atherosclerotic process. (Circulation. 2003;107:1383-1389.)

Key Words: endothelium ■ free radicals ■ xanthine oxidase ■ NADPH oxidase ■ coronary disease

Increased inactivation of nitric oxide (NO) by superoxide anions (O$_2^-$) is thought to contribute to impaired endothelium-dependent vasodilation in patients with coronary artery disease (CAD). Recent evidence suggests that reduction of vascular NO availability may have important prognostic implications in patients with CAD at least in part as a result of loss of antitherogenic properties of NO. These observations raise the question of what enzymatic sources contribute to increased radical formation in patients with CAD.

In experimental atherosclerosis, xanthine oxidase (XO) and NAD(P)H oxidase have been identified as major vascular O$_2^-$-forming enzyme systems; however, whether the activities of these pro-oxidant enzymes are increased in the arterial wall of patients with CAD remains to be determined. The present study was designed to characterize vascular XO and NAD(P)H oxidase activity in patients with CAD by using electron spin resonance spectroscopy (ESR). XO and NAD(P)H oxidase activities were determined in coronary artery specimens of patients with CAD and controls without atherosclerotic disease. To elucidate the contribution of XO to oxidative stress in vivo, we determined endothelium-bound xanthine oxidase (eXO) activity in patients with CAD and age-matched controls and in young individuals with hyper-
cholesterolemia (ie, in an early phase of the atherosclerotic disease process). To address the functional relevance of eXO in vivo, eXO activity was related to endothelium-dependent vasodilation of the radial artery and, in particular, to the portion of flow-dependent, endothelium-mediated vasodilation (FDD) inhibited by oxygen free radicals, as determined by the effect of the antioxidant vitamin C on FDD.

**Methods**

**Measurement of Vascular Xanthine and NAD(P)H Oxidase Activity With ESR: Ex Vivo Protocol**

Human coronary artery specimens were collected at autopsy from 10 patients with CAD (6 men; aged 60±5 years) and 10 age-matched control subjects (5 men; aged 54±5 years). Deceased patients were kept for the first 2 hours at room temperature and then for 23±5 hours at 4°C until autopsy. Control subjects had normal coronary arteries without histological evidence of denudation of the endothelium, infiltration of leukocytes, or foam cell formation. Further, the control subjects had no pathomorphological evidence or history of hypertension, diabetes mellitus, hypercholesterolemia, or smoking, as assessed by careful examination of patient records and contact with the primary physician. Control subjects died from diseases not related to atherosclerosis (traffic accident, n=3; pulmonary embolism, n=3; brain tumor, n=2; leukemia, n=1; and pneumonia, n=1).

To determine whether activities of XO and NAD(P)H oxidase are stable in vascular tissue for 36 hours at 4°C, we obtained specimens of umbilical veins that were handled in a similar manner as deceased patients (ie, umbilical vein specimens were kept at room temperature for 2 hours, then stored for 48 hours at 4°C and frozen at −80°C. In these experiments, we observed no loss or gain of activity for the enzymes studied (before versus after 48 hours: XO, 2.5±0.4 versus 2.3±0.3 nmol O2/µg protein; NADPH, 2.6±0.5 versus 2.8±0.3 nmol O2/µg protein; and NADH, 2.8±0.4 versus 2.6±0.3 nmol O2/µg protein; n=5).

Frozen coronary artery specimens were homogenized in 10 vol of 50 mMol/L Tris-Cl buffer (pH 7.4) containing 0.1 mMol/L EDTA, 0.1 mMol/L EGTA, and a set of antiproteolytic agents (1 mMol/L phenylmethyl sulfonl fluoride, 0.1 mMol/L bestatin, 1 mMol/L pepstatin, and 2 µMol/L leupeptin). Samples were centrifuged at 750g for 10 minutes (4°C), and supernatants were stored on ice until use. In some experiments, homogenates were separated into soluble (cytosolic) and particulate (membrane) fractions by ultracentrifugation (50 000g for 60 minutes at 4°C). Protein analysis was performed with the Bio-Rad DC Protein Assay. XO and NAD(P)H oxidase activity was determined by ESR using the spin trap 1-hydroxy-3-carboxy-pyrolidin-2-one (CP-H; Alexis Cooperation). ESR measurements were performed at room temperature with an EMX ESR spectrometer (Bruker Instruments Inc) with a TM110 cavity. The instrument settings were as follows: field center, 3497 G; field sweep 3; brain tumor, n=3; pulmonary embo-

**Measurement of eXO Activity In Vivo**

XO is localized and bound to the surface of endothelial cells by glycosaminoglycans1–16 and is released from endothelium into plasma by heparin bolus injection,13 allowing determination of eXO activity in humans in vivo. For measurement of plasma XO at baseline, 2 arterial (brachial artery) and 2 venous (ante-cubital vein) blood samples were drawn. Then, 5000 IU of heparin were injected into the brachial artery, and blood samples were drawn from the antecubital vein of the same arm (1, 3, 5, 7, 10, 15, and 20 minutes after heparin) into EDTA-containing vacuum tubes. Blood samples were centrifuged immediately (1500g for 15 minutes at 4°C), and plasma was stored at −80°C. The instrument settings for ESR measurements and the reagents used were the same as described above for in vitro measurements. The intensity of ESR spectra was quantified, and the ESR signal of plasma without xanthine (obtained for each sample) was subtracted. The coefficient of variation for determination of eXO activity in humans in vivo was 8.3%. In preliminary experiments, we found that plasma XO activity was completely inhibited by oxypurinol (1 mMol/L) or after boiling the sample, proving that xanthine-driven O2− formation was specifically mediated by XO. The activity of eXO (O2−/µl plasma per min) was calculated as area under the curve of the increase of plasma XO activity within 20 minutes after heparin bolus injection.

In 15 patients with CAD and 7 control subjects, endothelium-bound xanthine dehydrogenase (eXDH) activity was determined with a spectrophotometric assay.17 In brief, plasma samples were added on ice to reaction buffer (pH 7.4) containing 50 mMol/L potassium phosphate, 1 mMol/L EDTA, and 0.5 mMol/L nicotinamide adenine dinucleotide (NAD+). The reaction was started by adding xanthine (100 µMol/L), and NADH formation was monitored at 340 nm for 5 minutes at 37°C. Plasma eXDH activity (U·mL−1·min−1) was calculated as area under the curve of the increase of XDH activity within the first 20 minutes after heparin bolus injection. A calibration curve was performed with known amounts of NADH to calculate eXDH activity; 1 U was defined corresponding to the formation of 1 µmol of NADH per min. The coefficient of variation for determination of eXDH activity was 11.4%. Maximum increase of plasma eXDH activity was observed within the first 10 minutes after heparin bolus injection. Importantly, formation of NADH was completely abolished after preincubation of the samples with 1 mMol/L oxypurinol.

**Measurement of FDD**

Radial artery diameters were measured with high-resolution ultrasound (ASULAB). This method is well established in our laboratory,3–19 has an excellent reproducibility and variability, and was used as described in detail recently.16 Blood flow velocity was recorded continuously, and radial artery diameter was determined

<table>
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<tr>
<th>Characteristic</th>
<th>Protocol 1</th>
<th>Protocol 2</th>
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<tr>
<td>Controls</td>
<td>CAD</td>
<td>Controls</td>
</tr>
<tr>
<td>(n=10)</td>
<td>(n=21)</td>
<td>(n=6)</td>
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<tr>
<td>Age, y</td>
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<td>Mean blood pressure, mm Hg</td>
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<td>LDL cholesterol, mg/dL</td>
<td>120±3</td>
<td>166±13</td>
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<tr>
<td>Creatinine, µmol/L</td>
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<td>83±3</td>
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<tr>
<td>Body weight, kg</td>
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<td>86±1</td>
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<tr>
<td>Height, cm</td>
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Values are mean±SEM. HC indicates hypercholesterolemia. *P<0.05 vs controls.
every 30 seconds until stable baseline conditions were obtained (~30 minutes). Then, a wrist arterial occlusion (8 minutes) was performed, and FDD in response to reactive hyperemic blood flow was assessed at baseline and after intra-arterial infusion of vitamin C (25 mg/min for 10 minutes). Blood flow and diameter data reported for vitamin C represent measurements during the last minute of each infusion. All measurements were recorded, and vessel diameter and blood flow velocity were subsequently analyzed by 2 investigators unaware of the sequence of interventions.

In Vivo Protocol 1: CAD
FDD and eXO activity were measured in 21 patients with angiographically documented, stable CAD (left ventricular ejection fraction >45%) and 10 age-matched control subjects (Table). Patients with an acute coronary syndrome; diabetes mellitus; uncontrolled hypertension; hematological, renal, or hepatic dysfunction; heparin therapy within the last 48 hours; or patients taking antioxidant vitamin supplements were excluded. A total of 15 of the 21 studied patients with CAD had a serum LDL cholesterol <160 mg/dL.

Figure 1. A, XO activity in coronary arteries from patients with CAD and controls. XO activity was determined in homogenates (total) and in subcellular fractions (membrane and cytosolic). *P<0.05 vs without oxypurinol; #P<0.05 vs membrane fraction. B, NADPH- and NADH-driven O$_2^\cdot$ formation in homogenates and subfractions of coronary arteries from patients with CAD and controls. #P<0.05 vs membrane fraction.

Controls had no cardiovascular risk factors, as determined by a careful history, physical examination, and laboratory analysis. Vasovactive medications were withheld and both alcohol and caffeine were prohibited for at least 12 hours before the study. Written informed consent was obtained for all subjects, and the protocol was approved by the local Ethics Committee.

In Vivo Protocol 2: Hypercholesterolemia
FDD and eXO activity were determined in 6 young untreated individuals with asymptomatic familial hypercholesterolemia (type IIa) and 6 age-matched controls (Table). eXO activity was determined after heparin bolus injection (5000 IU) into the antecubital vein instead of the brachial artery, and FDD was measured as described above. The effect of vitamin C on FDD was not determined to avoid arterial puncture in young healthy individuals.

Statistical Analysis
All data are expressed as mean±SEM. To compare data between different groups, ANOVA was used to compare repeated measure-
Results

XO- and NAD(P)H Oxidase–Mediated \( \mathrm{O}_2^- \) Formation in Coronary Arteries

In coronary arteries of patients with CAD, both XO- and NAD(P)H oxidase–mediated \( \mathrm{O}_2^- \) formation were significantly increased compared with control vessels from age-matched subjects without CAD (Figure 1). Xanthine-driven \( \mathrm{O}_2^- \) formation was abolished by oxyipurinol (1 mmol/L), a specific inhibitor of XO (Figure 1A), whereas NAD(P)H-mediated \( \mathrm{O}_2^- \) formation was not affected by oxyipurinol (CAD with/without oxypurinol: NADPH, 12±2 versus 11±3 nmol \( \mathrm{O}_2^- / \mu \mathrm{g} \) protein; NADH, 11±1 versus 10±3 nmol \( \mathrm{O}_2^- / \mu \mathrm{g} \) protein; controls with/without oxypurinol: NAD(P)H, 9±1 versus 8±3 nmol \( \mathrm{O}_2^- / \mu \mathrm{g} \) protein; NADH, 7±1 versus 8±2 nmol \( \mathrm{O}_2^- / \mu \mathrm{g} \) protein; data not shown). Subcellular fractionation of vessel homogenates using ultracentrifugation revealed that XO (Figure 1A) and NAD(P)H oxidase-mediated \( \mathrm{O}_2^- \) formation was largely located within the membrane fraction (Figure 1B).

In Vivo Protocol 1: eXO Activity and FDD in Patients With CAD and Controls

Injection of 5000 IU heparin caused a 2- to 3-fold greater increase of plasma XO activity in patients with CAD compared with control subjects (25±4 versus 9±1 nmol \( \mathrm{O}_2^- / \mu \mathrm{L} \) plasma per min; \( P<0.05; \) Figure 2). Subgroup analysis revealed that under normocholesterolemic conditions (LDL-cholesterol <160 mg/dL), eXO activity was still significantly increased in patients with CAD compared with healthy control subjects (23±5 versus 9±1 nmol \( \mathrm{O}_2^- / \mu \mathrm{L} \) per min; \( P<0.05; \) data not shown). FDD, defined as percent increase in radial artery diameter after wrist occlusion, was substantially reduced in patients with CAD compared with control subjects (Figure 3). Infusion of the antioxidant vitamin C improved FDD in patients with CAD and had no effect in control subjects (Figure 3).

Forearm blood flow at rest (CAD versus controls: 35±6 versus 29±4 mL/min) and at maximal reactive hyperemia (102±15 versus 89±5 mL/min) was similar in patients with CAD and control subjects. Systemic blood pressure and heart rate did not change during the experimental protocol (data not shown).

In patients with CAD, there was an inverse relationship between eXO activity and FDD (\( r=-0.55; P<0.05; \) Figure 4A) and a positive relationship between eXO activity and the effect of vitamin C on FDD, representing the portion of FDD inhibited by oxygen free radicals (\( r=0.54; P<0.05; \) Figure 4B).

XDH activity after heparin bolus injection was also increased in patients with CAD (2.0±0.4 U · mL\(^{-1} \) · min\(^{-1}\)) compared with controls (0.6±0.3 U · mL\(^{-1} \) · min\(^{-1}\); \( P<0.05; \) data not shown).

[Figure 2: A. Increase of XO activity in plasma after heparin bolus injection (5000 IU) in patients with CAD (n=21) and control subjects (n=10). Increase of plasma XO activity was determined as difference between plasma XO activity before and after heparin injection. At time 0, difference between 2 baseline measurements is shown. Increase of plasma XO activity was significant from 3 minutes after injection of heparin (\( P<0.05 \) vs baseline). B. Representative ESR spectra of xanthine-induced CP formation in a plasma sample of a control subject and a patient with CAD before and 5 minutes after heparin injection.]

[Figure 3: Change in radial artery diameter (%) during reactive hyperemia (FDD) after wrist occlusion in patients with CAD (n=21) and age-matched control subjects (n=10) showing effect of intra-arterial infusion of the antioxidant vitamin C (25 mg/min for 10 minutes).]
In Vivo Protocol 2: eXO Activity and FDD in Young Individuals With Hypercholesterolemia

eXO activity was substantially increased in young individuals with hypercholesterolemia compared with age-matched control subjects (eXO: 33±6 versus 11±2 nmol O$_2$·/µl plasma per min; P<0.05; Figure 5B). FDD was reduced in individuals with hypercholesterolemia (9.1±0.3% versus 11.8±0.6%; P<0.05).

Discussion

The salient findings of the present study are as follows. (1) In coronary arteries of patients with CAD, XO and NAD(P)H oxidase activity are increased compared with control subjects without atherosclerotic disease. (2) In vivo, eXO activity is increased in patients with CAD compared with age-matched controls and is inversely related to endothelium-dependent vasodilation, suggesting that increased activity of XO contributes to endothelial dysfunction in patients with CAD. (3) In patients with CAD, eXO activity is closely related to the effect of vitamin C on NO-mediated vasodilation, supporting the concept that increased XO-mediated O$_2$· formation contributes to reduced bioavailability of NO within the arterial wall. (4) In young asymptomatic individuals with familial hypercholesterolemia, eXO activity is increased by >200% compared with age-matched controls, suggesting that activation of vascular XO represents an early mechanism contributing to increased radical formation and endothelial dysfunction in the atherosclerotic disease process.

Increased inactivation of NO by O$_2$· anions contributes to the reduction of vascular NO availability in patients with CAD. Increased evidence supports the concept that this loss of NO bioavailability not only leads to impaired endothelium-dependent vasodilation, but may have prognostic implications. In the present study, we used ESR to characterize the activity of vascular radical-forming enzyme systems in patients with CAD, young individuals with hypercholesterolemia, and age-matched controls. We observed a substantial increase of XO activity, a major source of O$_2$·, in coronary arteries from patients with CAD compared with age-matched control subjects. Furthermore, eXO activity, released from the endothelium by heparin bolus injection, was increased by >2-fold in patients with CAD compared with age-matched control subjects and was inversely related to FDD. These results are in line with recent experimental observations in hypercholesterolemic rabbits, demonstrating a dramatic reduction of vascular O$_2$· formation and improved endothelium-dependent vasodilation after inhibition of XO. Furthermore, release of XO by heparin improved NO-mediated vasodilation in hypercholesterolemic rabbits, supporting the concept that the observed inverse relationship between heparin-releasable XO activity and FDD in patients with CAD reflects a causal role of XO for endothelial dysfunction in CAD.

We also observed a positive relationship between XO activity and the portion of FDD inhibited by oxygen free
 radicals (ie, the effect of vitamin C on FDD) in patients with CAD. This finding suggests that elevated XO activity contributes to increased vascular oxidant stress in patients with CAD. Of note, in a recent study by Heitzer et al,2 the effect of vitamin C on endothelium-dependent vasodilation of peripheral arteries was shown to be an important prognostic indicator in patients with CAD.

To determine if the increase of vascular XO activity is an early event in the development of human atherosclerosis, we studied young individuals with familial hypercholesterolemia. In these subjects, eXO activity was increased by >200% compared with age-matched controls. This finding is in line with the observations of Cardillo et al,22 who demonstrated that inhibition of XO improved endothelium-dependent vasodilation in subjects with hypercholesterolemia.

The present study was not designed to elucidate mechanisms responsible for increased vascular XO activity in patients with CAD. The observations of the present study, however, suggest that the total activity of XDH/XO bound to the endothelium is increased in patients with CAD. Experimental studies have demonstrated that inflammatory cytokines, such as interferon-γ, can upregulate gene expression of XDH in endothelial cells, resulting in increased endothelial XO activity.23 This could contribute to the observed increase of XDH/XO in patients with CAD because increased secretion of interferon-γ by activated T-lymphocytes has been demonstrated in human atherosclerotic arteries.24 Furthermore, experimental studies support the concept that activation of the renin-angiotensin system may contribute to increased vascular XO activity, because inhibition of XO could markedly improve endothelium-dependent vasodilation in renin-angiotensinogen–overexpressing rats.25

During the past decade, several O₂⁻-forming enzyme systems using NAD(P)H as a cofactor have been identified in vascular cells that may contribute to NADPH-dependent O₂⁻ production, such as the vascular isoforms of the leukocyte xanthine oxidase,26 XO, cytochrome-P45028 and the uncoupled NO synthase. In the present study, we observed a substantial increase of NADH-/NADPH-stimulated O₂⁻ formation in coronary arteries from patients with CAD, supporting the concept that increased NAD(P)H oxidase activity also contributes to increased vascular oxidant stress in patients with CAD. These findings are in line with recent observations that the expression of NAD(P)H oxidase subunits, such as p22phox, is increased in atherosclerotic coronary arteries.26,29 Increased O₂⁻ formation by NAD(P)H oxidase may also contribute to impaired endothelium-dependent vasodilation. Guzik et al30 demonstrated an inverse relationship between NAD(P)H oxidase activity and endothelium-dependent vasodilation in human saphenous veins obtained from patients with coronary artery disease and identified risk factors.

In conclusion, the present study demonstrates that both vascular XO and NAD(P)H oxidase activity are increased in patients with CAD. In addition, XO activity is increased in young asymptomatic individuals with hypercholesterolemia, suggesting that activation of vascular XO represents an early event in the development of endothelial dysfunction in humans. Therapeutic interventions directed toward inhibition of these 2 enzyme systems may prove beneficial in treatment of vascular disease.

**Study Limitations**

In the present study, xanthine- and NAD(P)H-mediated O₂⁻ formation was determined in human coronary arteries that were obtained within the first 36 hours post mortem. The possibility that the activity of xanthine- and NAD(P)H oxidase is unpredictably altered over time cannot be definitively excluded. Accordingly, the findings of the present investigation will have to be validated in future studies using different approaches.

**References**


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_Circulation_. 2003;107:1383-1389; originally published online March 3, 2003; doi: 10.1161/01.CIR.0000056762.69302.46

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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