Superoxide Generation in Directional Coronary Atherectomy Specimens of Patients With Angina Pectoris

Important Role of NAD(P)H Oxidase

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Objective—NADH/NADPH oxidase is an important source of reactive oxygen species (ROS) in the vasculature. Recently, we demonstrated that p22phox, an essential component of this oxidase, was expressed in human coronary arteries and that its expression was enhanced with the progression of atherosclerosis. The present study was undertaken to investigate its functional importance in the pathogenesis of coronary artery disease. For this aim, the expression of p22phox, the distribution of oxidized low density lipoprotein (LDL), and the generation of ROS in directional coronary atherectomy (DCA) specimens were examined.

Methods and Results—DCA specimens were obtained from patients with stable or unstable angina pectoris. The distribution of p22phox and of oxidized LDL was examined by immunohistochemistry. The generation of superoxide in DCA specimens was assessed by the dihydroethidium method and lucigenin-enhanced chemiluminescence. ROS were closely associated with the distribution of p22phox and oxidized LDL. Not only inflammatory cells but also smooth muscle cells and fibroblasts generated ROS. There was a correlation between ROS and the expression of p22phox or oxidized LDL. The generation of ROS was significantly higher in unstable angina pectoris compared with stable angina pectoris.

Conclusions—ROS generated by p22phox-based NADH/NADPH oxidase likely mediate the oxidative modification of LDL and might play a major role in pathogenesis of atherosclerotic coronary artery disease. (Arterioscler Thromb Vasc Biol. 2002;22:1838-1844.)

Key Words: atherosclerosis ■ free radicals ■ coronary disease

Disruption of unstable plaques with superimposed coronary thrombosis is the main cause of acute coronary syndrome, including unstable angina pectoris (UAP) and acute myocardial infarction. Mechanisms of plaque instability and factors to determine plaque vulnerability remain to be elucidated. However, there is accumulating evidence that a family of matrix metalloproteinases (MMPs) plays a crucial role in the determination of strength and vulnerability of atherosclerotic plaques.1 Recently, it has been reported that reactive oxygen species (ROS) activate MMPs.2,3 Therefore, oxidative stress in the vasculature plays an important role not only in atherogenesis but also in the instability of atherosclerotic plaques.

NADH/NADPH oxidase is a major source of ROS in vascular cells, including smooth muscle cells (SMCs), endothelial cells, and adventitial fibroblasts.4–6 This oxidase system was originally identified as the defense against exogenous microorganisms in phagocytes. The phagocytic NADH/NADPH oxidase is composed of at least 6 components: plasma membrane–spanning cytochrome b558 composed of gp91phox and p22phox, 3 cytosolic components (p67phox, p47phox, and p40phox), and rac, a small G protein. Although intensive investigations have been conducted to identify the vascular NADH/NADPH oxidase, its molecular characterization still remains unclear. Recently, various homologues of...
phagocytic gp91
phox, ie, the NOX family, have been cloned, and investigations regarding with their functional roles in cardiovascular diseases are now ongoing. On the other hand, Ushio-Fukai et al reported that transfection of anti-sense oligonucleotide of p22 phox suppressed ROS generation in vascular SMCs. Furthermore, we and another group demonstrated that p22 phox was expressed in all major cellular components of the human vessel wall: endothelial cells, SMCs, macrophages, and fibroblasts. These findings indicate that p22 phox is likely a common component of vascular and phagocytic NADH/NAPDH oxidase systems. These observations led us to hypothesize that p22phox is a key molecule in the pathogenesis of atherosclerotic coronary artery disease and acute coronary syndrome. To examine the functional role of p22phox-based NADH/NAPDH oxidase systems, we investigated the expression of p22phox in atherosclerotic plaques obtained from patients with UAP or SAP. The target sites of atherectomy were judged as culprit lesions by 99m Tc-tetrofosmin (exercise or ATP-loading) scintigraphy. In the case of patients with UAP, 99m Tc-tetrofosmin (rest) scintigraphy was carried out for the determination of culprit lesions. No significant differences existed between UAP and SAP in age, sex, and the incidence of risk factors. The degree of angiographic stenosis was similar as well. After the DCA procedure, atherectomy tissues were immediately embedded in OCT compound (SAKURA Finetechnical Co.) in liquid nitrogen and stored at −80°C.

Methods

DCA Specimens From Patients With Angina Pectoris

The present study was approved by the hospital ethics committee, and informed consent was obtained from all patients. Coronary atherectomy specimens were obtained from 36 consecutive angina patients undergoing DCA at Kobe University Hospital and Miki City Hospital. Clinical profile and angiographic data of patients are shown in the Table. Sixteen patients were classified as IIB. Ten patients were classified as IIB, 4 patients were classified as IIIC, and 2 patients were classified as IIIC. The angiographic appearances before DCA were evaluated by the classification reported by Ambrose et al: concentric narrowing; type I eccentric narrowing (asymmetric narrowing with smooth borders and broad neck); type II eccentric narrowing (asymmetric with a narrow neck or irregular border or both); and multiple irregular coronary narrowing in series. The target sites of atherectomy were judged as culprit lesions by 99m Tc-tetrofosmin (exercise or ATP-loading) scintigraphy. In the case of patients with UAP, 99m Tc-tetrofosmin (rest) scintigraphy or 123I-BMIPP scintigraphy was carried out for the determination of culprit lesions. No significant differences existed between UAP and SAP in age, sex, and the incidence of risk factors. The degree of angiographic stenosis was similar as well. After the DCA procedure, atherectomy tissues were immediately embedded in OCT compound (SAKURA Finetechnical Co.) in liquid nitrogen and stored at −80°C.

Detection of In Situ Generation of ROS

To detect in situ generation of ROS in DCA specimens, fluorescence microtopography with dihydroethidium was performed as previously described. Briefly, unfixed frozen samples were cut into 5 μm-thick sections and placed on glass slides. Dihydroethidium (10 μmol/L) was applied to each tissue section, and then the sections were coverslipped. The slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. The image of dihydroethidium was obtained by a laser scanning confocal imaging system (MRC-1024, BioRad) with a 585-nm long-pass filter. Generation of ROS was demonstrated by red fluorescence labeling.

Lucigenin-Enhanced Chemiluminescence

The generation of superoxide from DCA specimens or rabbit aortas was also estimated by using lucigenin-enhanced chemiluminescence as previously described. The light reaction between superoxide and lucigenin was detected in a chemiluminescence reader (BLR-201, Aloka). The assay solution contained 50 mmol/L HEPES (pH 7.4), 135 mmol/L NaCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 5 mmol/L KCl, 5.5 mmol/L glucose, and 5 μmol/L lucigenin as the electron donor.
Immunofluorescence

Immunofluorescence experiments were performed as previously described.6 Unfixed frozen samples were cut from a given sample and air-dried onto slides. Additional serial cryostat sections were stained with hematoxylin and eosin for analysis of morphological details by light microscopy. The tissue slices were fixed with 100% acetone at −20°C for 10 minutes. The sections were incubated with BSA (Dako LSAB kit, Dako A/S) for 60 minutes at room temperature and then incubated with primary antibody overnight at 4°C. The primary antibodies used in the present study were mouse monoclonal anti-human oxidized LDL antibody (DLH3) and rabbit polyclonal anti-human p22phox antibody that was against synthetic peptide corresponding to its C-terminal region (residues 175 to 194). DLH3 recognized oxidized phosphatidylcholine (ox-PC) and protein modified by ox-PC. The specificity of these antibodies has been reported previously.17,18 Texas red–conjugated anti-immunoglobulin was applied as secondary antibody. The samples were examined by the laser scanning confocal imaging system. The presence of p22phox or oxidized LDL was demonstrated by red immunofluorescence labeling.

Double labeling was performed as previously described.6 The cell-specific antibodies were mouse monoclonal anti-human CD68 antibody (clone KP-1, Dako A/S) for macrophages, anti-smooth muscle α-actin antibody (clone 1A4, Dako A/S) for SMCs, anti-prolyl 4-hydroxylase antibody (clone 5B5, Dako A/S) for fibroblasts, and UCHL-1 antibody (Dako A/S) for T lymphocytes. In case of double labeling of dihydroethidium and immunofluorescence, at first the detection of ROS by dihydroethidium was performed, and then immunofluorescence was sequentially performed by using the same samples. FITC-conjugated anti-immunoglobulin was used as the secondary antibody for immunofluorescence. A laser scanning confocal imaging system examined these samples. Red and green labelings show dihydroethidium (ROS) and immunofluorescence (p22phox, oxidized LDL, or cell-specific markers), respectively.

Quantification and Statistical Analysis

To compare fluorescence signals between different specimens, semiquantitative analysis was performed. All DCA specimens were digitized by a digital camera. The total area of each section and the surface area occupied by ROS, p22phox, oxidized LDL, or cell marker–positive cells were outlined on computer-aided planimetry with the use of image analysis software (Macscope, Mitani Co). The fluorescence areas were measured automatically by using gray-scale detection with a fixed threshold. The relative expression of ROS, p22phox, oxidized LDL, or cell markers was expressed as the ratio of positive area to total surface area in each specimen.

In a separate experiment, dihydroethidium staining and lucigenin-enhanced chemiluminescence were simultaneously applied for detection of superoxide production from aortas of Japanese white rabbits or Watanabe heritable hyperlipidemic rabbits to examine the validity of quantification of ROS. As shown in Figure 1D, there was a significant correlation between values measured by lucigenin-enhanced chemiluminescence and dihydroethidium staining. The intraobserver and interobserver variations were assessed by the method of Bland and Altman.19 The intraobserver and interobserver comparisons revealed high correlations (r=0.90 to 0.95), and there was no significant variation in intraobserver and interobserver data. Data are expressed as mean±SEM. Differences between 2 groups were analyzed by the Mann-Whitney nonparametric test and were considered significant at P<0.05. Linear regression analyses were performed between ROS-positive and p22phox-positive, oxidized LDL-positive and ROS-positive, or cell marker–positive and p22phox-positive areas.

Results

Detection of ROS and Distribution of Oxidized LDL and p22phox in DCA Specimens

All atherectomy specimens were examined by hematoxylin-eosin stain. Various kinds of pathological changes, ie, hypercellular region, extracellular lipid deposits, fibrous tissues, and coronary thrombosis, were observed in DCA specimens. In situ generation of ROS in DCA specimens was detected by the dihydroethidium method, as shown in Figure 1A through 1F. Fluorescence signals of dihydroethidium were suppressed by incubation of Cu/Zn superoxide dismutase (SOD) and MnTBAP.

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cellular lesion, which was obtained from a patient with UAP, had intense signals of ROS (Figure 2A and 2B). Immunohistochemical examination of the serial section of this sample demonstrated the intense expression of p22phox as well as oxidized LDL (Figure 2C and 2D). In case of the DCA specimen with much fibrous tissue, the production of ROS was very weak (E and F). Similarly, the expression of p22phox as well as oxidized LDL was low (G and H).

To further examine the relationship among ROS generation, p22phox, and oxidized LDL, double staining was performed. Figure 3A through 3C shows representative samples of double labeling of dihydroethidium and immunofluorescence for p22phox. There was a considerable overlapping of ROS and p22phox. Figure 3E through 3G shows representative samples of double labeling of dihydroethidium and immunofluorescence for oxidized LDL. The distribution of oxidized LDL was partially associated with the generation of ROS. Furthermore, semiquantitative analysis was performed to examine the correlation between ROS and p22phox or oxidized LDL-positive cells. As shown in Figure 3D and 3H, the amounts of ROS-positive cells were significantly correlated with the expression of p22phox. Furthermore, there is a significant correlation between ROS and oxidized LDL-positive cells.

**Cell Type of ROS-Generating Cells and Comparison of SAP and UAP**

To identify the types of ROS-generating cells, double labeling with cell-specific markers was performed. As shown in Figure II (please see www.ahajournals.org), macrophages and fibroblasts were positive for the fluorescence signal of dihydroethidium. Interestingly, some SMCs and a few T lymphocytes generated ROS in DCA samples.

The generation of ROS in DCA specimens was compared between UAP and SAP by a lucigenin-enhanced chemiluminescence method. The generation of superoxide was significantly higher for UAP than for SAP (7.3 ± 1.2 [n = 6] and 3.2 ± 1.0 [n = 6] cpm/mg protein, respectively).

Plaque instability is largely influenced by the extent of inflammation, and p22phox is reported to be highly expressed in inflammatory cells. Therefore, the enhanced generation of superoxide in UAP might be due to the increased inflammatory cells. To investigate the relationship between the expression of p22phox and inflammation, semiquantitative analysis was performed to examine the correlation between p22phox and CD68, a marker for macrophages. As shown in online Figure III (please see www.ahajournals.org), there was a significant correlation between CD68 and p22phox.

Next, to compare the amounts of p22phox-expressing SMCs in UAP versus SAP, double staining of p22phox and α-actin was semiquantitatively analyzed. As shown in Figure 4, the amounts of cells that were positive for both p22phox and α-actin in DCA specimens were significantly higher for UAP than for SAP. Thus, not only the increased infiltrating macrophages but also the enhanced expression of p22phox in SMCs contributed to a higher expression of NADH/NADPH oxidase p22phox in UAP.

**Discussion**

The present study is the first demonstration of in situ ROS generation of DCA specimens from patients with angina pectoris. The production of ROS was closely associated with the expression of p22phox, indicating the functional importance of p22phox-based NADH/NADPH oxidase. ROS-generating cells in DCA specimens were mainly accumulating macrophages. However, in addition to macrophages, some SMCs,
fibroblasts, and T lymphocytes generated ROS. Furthermore, the generation of ROS was overlapped with the distribution of oxidized LDL and p22phox. These findings suggest that ROS generated by p22phox-based NADH/NADPH oxidase likely mediate the oxidative modification of LDL. Although there was no significant difference of angiographic stenosis, the generation of ROS was significantly higher in UAP patients compared with SAP patients. These findings indicate that oxidative stress in the coronary bed seems to influence the disease state of angina pectoris rather than the degree of coronary stenosis. The majority of acute coronary syndrome has been reported to occur at the site with <70% stenosis, according to angiographic studies on patients before coronary events. On the basis of data suggesting an interrelation between ROS and p22phox, the enhanced oxidative stress by p22phox-based NADH/NADPH oxidase might play an important role in the vulnerability of atherosclerotic plaques.

The stability of an atherosclerotic plaque largely depends on the structural integrity of its fibrous cap, which is composed of mainly extracellular matrix component. Therefore, the dissolution of the extracellular matrix by MMPs provides a likely contributing mechanism of plaque rupture. Rajagopalan et al reported that ROS induced the activation of MMPs. Furthermore, we found that lysophosphatidylcholine, a major component of oxidized LDL, increased the release of MMP-2 through the activation of the endothelial NADH/NADPH oxidase. Taken together, it is interesting to speculate that MMPs might be activated by ROS generated by p22phox-based NADH/NADPH oxidase in coronary arteries and that their activation, in turn, contributes to pathogenesis of UAP.

The NADH/NADPH oxidase system had been originally described as the source of ROS in phagocytes, and it is a first line of defense against microorganisms. This oxidase is highly expressed and active in inflammatory cells, and the unstable plaques have been reported to contain advanced inflammation. In the present study, there was a correlation between CD68 and p22phox, as shown in online Figure III. Therefore, the higher expression of p22phox in UAP may be due to the enhanced infiltration of inflammatory cells. However, double staining of α-actin and p22phox demonstrated that p22phox-expressing SMCs were also increased in UAP (Figure 4). The regulatory mechanisms of p22phox expression in SMCs remain poorly understood; however, there is a possibility that activated SMCs in vulnerable plaques may express p22phox to a much higher degree. Recently, the 5′-flanking promoter region of the p22phox gene has been identified. There are many cis-acting transcriptional regulatory sites, suggesting that a variety of extracellular stimuli might be involved in the expression of this gene. The inflammatory cytokines and hemodynamic forces, such as shear stress and stretch force, might be mediators of the expression of p22phox.
nism whereby p22phox is upregulated in SMCs in coronary specimens of UAP remains to be elucidated.

The regulatory mechanisms of the enzymatic activity of vascular NADH/NADPH oxidase are not well defined, although several protein kinase–mediated pathways have been proposed. In the present investigation, the overlapping of p22phox and ROS was not complete; therefore, the expression of p22phox protein alone might not be sufficient for the production of superoxide, and its activation likely requires it. Several enzymatic origins other than NADH/NADPH oxidase have been proposed, such as cyclooxygenase, lipoxygenase, xanthine oxidase, myeloperoxidase, and NO synthase. Our results indicate the functional importance of NADH/NADPH oxidase; however, they do not deny roles of other oxidase systems.

Oxidative modification of LDL is involved in atherogenesis, and uptake of oxidized LDL by macrophages leads to the formation of foam cells. Oxidized LDL induces endothelial dysfunction, the expression of adhesion molecules, and apoptosis of SMCs.21,22 Previous investigations using DLH3 antibody demonstrated that oxidized LDL was localized in SMCs and macrophages in atherosclerotic arteries.23,24 Recently, Ehara et al23 demonstrated that the plasma level of oxidized LDL was increased in cases of acute coronary syndrome. Furthermore, they demonstrated that the surface area containing oxidized LDL–positive macrophages in the atherectomy specimens was significantly higher for UAP than for SAP.23 Our findings are consistent with theirs. In the present study, we extended their findings to the association of ROS and NADH/NAPDH oxidase. The molecules recognized by antibody DLH3 are ox-PC and proteins modified by ox-PC.18 Inasmuch as ox-PC and lyso-phosphatidylcholine are reported to cause endothelial dysfunction,25,26 these atherogenic lipids are key molecules that have the ability to promote atherosclerotic coronary artery diseases.

Several ways for the detection of ROS, eg, lucigenin-enhanced chemiluminescence, electron spin resonance, and the cytochrome c reduction method, have been reported. Each has merit and demerit regarding its sensitivity, specificity, and convenience to handle, and it is ideal to evaluate the generation of ROS by several different methods. In the present study, the generation of ROS was assessed by 2 different methods: microtopography with dihydroethidium and lucigenin-enhanced chemiluminescence. Both methods demonstrated the enhanced production of ROS in UAP. Although there are some criticisms such as the conversion reaction of dihydroethidium by cytochrome c, its validity has been confirmed by many previous reports.27,28 Furthermore, in a separate experiment involving the aortas of Japanese white rabbits and Watanabe heritable hyperlipidemic rabbits, we confirmed that there was a good correlation between values measured by microtopography with dihydroethidium and values measured by lucigenin-enhanced chemiluminescence (online Figure I).

Recently, Wagner et al29 reported that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors suppressed the assembly of the component of NADH/NADPH oxidase. It is interesting to speculate that HMG-CoA reductase inhibitors might make atherosclerotic plaques stable through the inhibition of NADH/NADPH oxidase. This inhibitory effect might contribute to beneficial effects of HMG-CoA reductase inhibitors on the cardiac events.

In conclusion, ROS generation in DCA specimens from patients with angina pectoris was detected by the dihydroethidium method. There is a significant association among ROS generation, p22phox, and oxidized LDL. The generation was significantly higher for UAP than for SAP. Given the
importance of ROS, p22\textsuperscript{phox}-based NADH/NADPH oxidase plays an important role in pathogenesis of coronary artery diseases, including acute coronary syndrome.

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**Figure I A.** Measurement of superoxide from rabbit aorta by dihydroethidium staining and lucigenin-enhanced chemiluminescence. Both methods were simultaneously applied for measurement of superoxide production from aortae of Japanese white rabbits or Watanabe heritable hyperlipidemic (WHHL) rabbits. There was a significant correlation between values measured by lucigenin-enhanced chemiluminescence and dihydroethidium staining.
Figure II A through D. Double labeling immunofluorescence to identify types of ROS-generating cells with cell type specific markers. Green fluorescence signals show cell specific markers. Anti-CD68 antibody (A), anti-α-actin antibody (B), anti-prolyl hydroxylase antibody (C), and anti-UCHL-1 antibody (D) were used as markers of macrophages, SMCs, fibroblasts, and T lymphocytes, respectively. Red fluorescence signals show in situ ROS generation detected by dihydroethidium fluorescence microtopography. Colocalization of cell specific markers and ROS signal is shown by yellow fluorescence. Macrophages were almost positive for fluorescence signal of dihydroethidium (A). Interestingly, some SMCs (B) and fibroblasts (C), and a few T lymphocytes (D) generated ROS in DCA samples. Results are representative of five experiments.
Figure III: Semi-quantitative analysis to show the correlation of CD68-positive cells with the expression of p22phox.