Induction of NAD(P)H Oxidase by Oxidized Low-Density Lipoprotein in Human Endothelial Cells

Antioxidative Potential of Hydroxymethylglutaryl Coenzyme A Reductase Inhibitor Therapy

Uwe Rueckschloss, MS; Jan Galle, MD; Juergen Holtz, MD; Hans-Reinhard Zerkowski, MD; Henning Morawietz, PhD

Background—Elevated oxidative stress and superoxide anion formation in vascular cells could promote conversion of LDL to atherogenic oxidized LDL (oxLDL), contributing to endothelial dysfunction and atherosclerosis. As a major source of vascular superoxide anion formation, an endothelial NAD(P)H oxidase, similar to the leukocyte enzyme, has been identified.

Methods and Results—To elucidate functional differences between NAD(P)H oxidases of endothelial cells and leukocytes, DNA sequences of endothelial NAD(P)H oxidase subunits were determined. Gp91phox cDNA sequence showed no difference between the 2 cell types. Endothelial p67phox cDNA sequence revealed 2 known polymorphisms, which do not affect NAD(P)H oxidase function. Next, we analyzed relative mRNA expression of NAD(P)H subunits in human umbilical vein endothelial cells (HUVECs) and leukocytes using a common cRNA standard in competitive reverse transcription–polymerase chain reaction. NAD(P)H oxidase subunits p22phox and p47phox are expressed at a similar level in both cell types, whereas p67phox (2.5%) and gp91phox (1.1%) are expressed at a much lower level in endothelial cells than in leukocytes. Differences of gp91phox expression in leukocytes and HUVECs correlate with differences in superoxide release. Gp91phox mRNA and endothelial superoxide anion formation are induced in response to oxLDL in HUVECs. Furthermore, a lower gp91phox mRNA expression was found in internal mammary artery biopsy samples of patients with coronary artery disease treated with HMG-CoA reductase inhibitors before coronary bypass surgery.

Conclusions—We conclude that oxLDL induces proatherosclerotic NAD(P)H oxidase expression and superoxide anion formation in human endothelial cells and an antioxidative potential of HMG-CoA reductase inhibition via reduction of vascular NAD(P)H oxidase expression. (Circulation. 2001;104:1767-1772.)

Key Words: atherosclerosis ■ endothelium ■ free radicals ■ lipoproteins

Oxidative stress and the generation of reactive oxygen species, including superoxide and nitric oxide (NO), are thought to play an important role in the regulation of proatherosclerotic and antiatherosclerotic processes in the vessel wall.1-3 Superoxide rapidly reacts with NO, resulting in peroxynitrite formation.5 Because NO is known to mediate antiatherosclerotic properties of the endothelium, eg, by inhibition of platelet aggregation,4 adhesion molecule expression,3 and vascular smooth muscle cell proliferation,6 the inactivation of NO by superoxide anions can contribute to the initiation of atherosclerosis. Furthermore, superoxide can regulate redox-sensitive vascular gene expression, eg, stimulating adhesion molecule expression, by changing the redox state of endothelial cells.7 In addition, elevated superoxide formation by vascular cells stimulates the conversion of LDL to the oxidatively modified LDL, oxLDL.8,9 The proatherosclerotic oxLDL can be internalized by endothelial cells and infiltrated macrophages, contributing to endothelial dysfunction and foam cell formation.

As a major source of superoxide anion generation in endothelial cells, an NAD(P)H oxidase, similar to the enzyme found in leukocytes, has been identified.10,11 In leukocytes, the NAD(P)H oxidase consists of 4 subunits. The subunits gp91phox and p22phox reside in the plasma membrane and form the cytochrome b558,12,13 whereas the 2 cytosolic subunits p67phox and p47phox are necessary for activation of the enzymatic complex, as demonstrated in cell-free reconstitution assays.14 Functional studies revealed 2 major differ-

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ences between the leukocyte and endothelial NAD(P)H oxidase systems. In contrast to leukocytes, the enzyme complex in endothelial cells prefers the substrate NADH over NADPH and releases superoxide anions at a lower rate.\textsuperscript{10,15} A substrate-binding site has been identified in the subunits gp91phox and p67phox.\textsuperscript{16,17} The molecular and structural basis for the functional differences between leukocytes and endothelial cells, however, remains to be elucidated.

In this study, we determined the DNA sequences of the putative substrate-binding subunits from human endothelial cells and compared them with the sequences from leukocytes. To gain further insight into the structural and functional differences between NAD(P)H oxidases of both cell types, we compared the level of NAD(P)H oxidase subunit expression in human leukocytes and endothelial cells using a common standard for all 4 subunits in competitive reverse transcription–polymerase chain reaction (RT-PCR). The mRNA level of NAD(P)H oxidase subunits was compared with superoxide release in both cell types. Furthermore, the effect of proatherosclerotic oxLDL on NAD(P)H oxidase expression and superoxide release in human endothelial cells was studied. To provide additional evidence that the in vitro findings take place in vivo, we determined the effect of lipid-lowering therapy by HMG-CoA reductase inhibitors on vascular expression of NAD(P)H oxidase in patients with coronary artery disease.

**Methods**

**Endothelial Cell Culture**

Cell culture reagents and chemicals were purchased from Sigma Chemical Co except when otherwise specified. Primary cultures of human umbilical vein endothelial cells (HUVECs) were isolated with collagenase IV as described previously.\textsuperscript{18,19} Human endothelial cell line ECV304 was purchased from ATCC and cultured in the described medium.

**Preparation of Leukocytes**

Human leukocytes were purified from blood anticoagulated with heparin by Ficoll density-gradient centrifugation.\textsuperscript{20} Leukocytes were subsequently diluted with PBS and sedimented at 1800g for 20 minutes. The leukocyte preparations contain $\sim$30% lymphocytes, 1% to 3% monocytes, and 67% to 69% granulocytes. Afterward, leukocytes were used for RNA preparation or cytochrome c reduction assay.

**Patients**

Distal remnant specimens of the left internal mammary artery (arteria thoracica interna) obtained after informed consent from 14 patients undergoing elective CABG surgery were used in this study. The use of human tissue was approved by the local ethics committee. HMG-CoA reductase inhibition therapy (lovastatin, pravastatin, or simvastatin; prescribed dosages by referring physicians were included in some experiments. Because NAD(P)H oxidase of leuko-

<table>
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<tr>
<th>Subunit</th>
<th>PCR Primer Sequences</th>
<th>PCR Protocol</th>
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<tr>
<td>p67phox</td>
<td>UP: 5'-GTTGTTGTTGAGGAGCGGCA-3'</td>
<td>95°C/30 s</td>
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<tr>
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<td>41 cycles</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>40 cycles</td>
</tr>
<tr>
<td>gp91phox</td>
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<tr>
<td></td>
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<td>44 cycles</td>
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UP indicates upstream primer; DP, downstream primer.

fraction, age, sex, height, or weight. In addition, no differences in concomitant therapy with ACE inhibitors (n = 3), calcium antagonists (n = 1), \( \beta \)-blockers (n = 6), diuretics (n = 2), NO donors (n = 11), heart glycosides (n = 1), or antidiabetics (n = 1) were found. No other lipid-lowering drugs were prescribed.

**DNA Sequence Analysis**

Total RNA from the human endothelial cell line ECV304 was used to amplify DNA fragments, including the complete coding regions of gp91phox and p67phox cDNA by RT-PCR. The DNA sequence of these RT-PCR fragments was determined by cycle sequencing with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) on an automated ABI 373A DNA Sequencer (ABI/Perkin-Elmer). Database searches of GenBank were performed with BLASTN and compared with the human leukocyte gp91phox\textsuperscript{12} and p67phox\textsuperscript{21} cDNA sequences.

**Quantification of NAD(P)H Oxidase Subunit mRNA Expression by Multistandard-Assisted RT-PCR**

Total RNA from HUVECs, leukocytes, and internal mammary artery biopsy samples was isolated by guanidinium thiocyanate/cesium chloride centrifugation as described.\textsuperscript{14} For comparison of mRNA expression of NAD(P)H oxidase subunits p67phox, p22phox, p47phox, and gp91phox in competitive RT-PCR, a common linker primer, PCR-generated, internal-deleted, and in vitro–transcribed multistandard cRNA was generated. DNA sequences of upstream primers and downstream PCR primers and PCR protocols are summarized in the Table. The identity of the amplified RT-PCR fragments was confirmed by DNA sequencing. Competitive RT-PCR was performed as previously described\textsuperscript{18,19} with the NAD(P)H oxidase multistandard or CD11c-specific primers.

**Cytochrome c Reduction Assay**

Cells were incubated at 37°C in assay buffer consisting of medium M199 without phenol red supplemented with 40 \( \mu \) mol/L cytochrome c and 500 \( \mu \) mol/L NG-nitro-L-arginine methyl ester to exclude ecNOS as a possible source of detected \( \text{O}_2^- \) generation. To evaluate the NAD(P)H oxidase–derived \( \text{O}_2^- \) generation, the flavin-containing enzyme inhibitor diphenylene iodonium (DPI, 100 \( \mu \) mol/L) was included in some experiments. Because NAD(P)H oxidase of leuko-
cytes can be maximally activated by protein kinase C stimulation, phorbol 12-myristate 13-acetate (PMA, 1 μmol/L) was used in a series of experiments comparing O$_2^-$ generation from leukocytes and HUVECs. At the indicated time points, aliquots of the supernatant were taken, absorption at 550 nm was determined, and blank was subtracted. The amount of O$_2^-$ generated was estimated by use of the millimolar extinction coefficient for reduced cytochrome c (29.5 L·mmol$^{-1}$·cm$^{-1}$). The O$_2^-$ generation was normalized versus protein concentration of the samples determined with the BCA Protein Assay Reagent (Pierce). The DPI-inhibited O$_2^-$ generation was estimated as the difference between samples with or without DPI in each group.

**OxLDL Studies**

LDL was isolated by sequential ultracentrifugation from human plasma. The oxidative modification of LDL with cupric ion was done as previously described. Confluent cultures of HUVECs were incubated with oxLDL, and subsequently the NAD(P)H oxidase expression and superoxide release was determined.

**Statistical Analysis**

Data are shown as mean±SEM. Statistical analysis was performed with the ANOVA procedure followed by the Bonferroni t test (multiple comparison) or Student’s t test (SigmaStat software, Jandel Corp). Differences were taken as statistically significant at a value of $P<0.05$.

**Results**

**Determination of Endothelial gp91phox and p67phox cDNA Sequence**

The complete coding regions of gp91phox and p67phox cDNA, both subunits reported to contain a binding site for NAD(P)H, were analyzed by DNA sequencing of RT-PCR–derived fragments from the endothelial cell line ECV304. For gp91phox, no structural basis for an altered substrate specificity between endothelial cells and leukocytes could be detected. In p67phox, 2 bp substitutions between leukocytes and endothelial cells (T$_{957}$→C, A$_{958}$→G) were detected. Of these substitutions, the first predicts a silent amino acid change (Leu$_{299}$→Leu), and the second predicts a conservative modification (Lys$_{328}$→Arg).

**Comparison of NAD(P)H Oxidase in Human Endothelial Cells and Leukocytes**

To gain further insight into the molecular and functional differences of NAD(P)H oxidase in both cell types, we analyzed the relative mRNA expression level of NAD(P)H subunits in primary cultures of HUVECs and leukocytes by use of a common cRNA standard for all 4 subunits in competitive RT-PCR (Figure 1). The subunits p22phox (45% of leukocytes) and p47phox (167% of leukocytes) are expressed in HUVECs and leukocytes at a similar level, whereas p67phox (2.5% of leukocytes) and gp91phox (1.1% of leukocytes) are expressed at a much lower level in endothelial cells than in leukocytes. Differences of gp91phox expression in leukocytes and HUVECs correlate well with the differences in corresponding superoxide release determined by DPI–inhibited superoxide anion formation in the cytochrome c reduction assay (Figure 2). The rate of superoxide anion formation in HUVECs corresponds to 1% of the maximal rate in leukocytes after stimulation with PMA. When the expressions of all 4 subunits in HUVECs were compared, gp91phox was found to be expressed at the lowest level. This provides evidence that gp91phox can be considered the limiting subunit of NAD(P)H oxidase in HUVECs, explaining the low rate of superoxide formation in endothelial cells.

**Induction of NAD(P)H Oxidase by OxLDL in Human Endothelial Cells**

Next, we analyzed the effect of oxLDL on the expression of limiting NAD(P)H oxidase subunit gp91phox in HUVECs. OxLDL induces 2-fold expression of gp91phox mRNA in HUVECs (maximum 1 hour) (Figure 3). The maximum of gp91phox induction was found after stimulation with 100 μg/mL oxLDL (Figure 4). The increased gp91phox expression at the mRNA level is accompanied by augmented endothelial superoxide anion formation after exposure to oxLDL (Figure 5).
Downregulation of Vascular Gp91phox mRNA Expression by HMG-CoA Reductase Inhibition Therapy in Patients With Coronary Artery Disease

Finally, we measured vascular gp91phox expression in internal mammary artery biopsy samples from patients with or without lipid-lowering therapy using HMG-CoA reductase inhibitors before coronary bypass surgery. Treatment of patients with HMG-CoA reductase inhibitors resulted in significant down-regulation of gp91phox mRNA expression in internal mammary arteries (Figure 6A). This lower gp91phox expression was not due to reduced levels of phagocytic cells, because in the same biopsies, mRNA expression of phagocytic cell marker CD11c was not different between the 2 groups of patients (Figure 6B). No significant correlation of gp91phox mRNA expression with other medications could be found in these patients.

Discussion

Vascular NAD(P)H oxidases, similar to the leukocyte enzyme complex, are considered a major source of superoxide anion formation in blood vessels. Functional studies of the vascular NAD(P)H oxidase revealed 2 major differences from the leukocyte enzyme complex. In most studies, NADH is proposed to be the preferred electron donor of the vascular enzyme complex. Furthermore, vascular NAD(P)H oxidase–dependent

Figure 3. Induction of gp91phox expression in HUVECs by oxLDL. Stimulation of HUVECs with 100 μg/mL oxLDL causes transient induction of gp91phox mRNA expression as determined by standard calibrated competitive RT-PCR. 
P<0.05.

Figure 4. Dose-dependent induction of gp91phox expression in HUVECs by treatment with oxLDL for 1 hour. gp91phox mRNA expression was determined by standard calibrated competitive RT-PCR. Maximal induction was detected at 100 μg/mL oxLDL. 
P<0.05.

Figure 5. Time-dependent induction of DPI-inhibited superoxide release in HUVECs by oxLDL. Incubation of HUVECs for 2 or 3 hours with 100 μg/mL oxLDL significantly induces DPI-inhibited O$_2^-$ formation as measured by cytochrome c reduction assay. 
P<0.05.

Figure 6. HMG-CoA reductase inhibitor therapy downregulates vascular gp91phox mRNA expression in internal mammary arteries of patients with coronary artery disease. Chronic treatment of patients with HMG-CoA reductase inhibitors before elective CABG surgery significantly reduces expression of gp91phox mRNA in internal mammary arteries (A). mRNA expression of phagocytic cell marker CD11c is not different in internal mammary artery segments between 2 groups of patients with or without HMG-CoA reductase inhibitor therapy (B). mRNA expression was quantified by standard calibrated competitive RT-PCR (in relative units). 
P<0.05.
superoxide anion formation was found to be rather constitutive at a much lower level than in leukocytes. To find a structural basis for the different substrate specificity of the endothelial NAD(P)H oxidase, the putative substrate-binding subunits gp91phox and p67phox were amplified and sequenced from the endothelial cell line ECV304. No difference in the complete coding region of gp91phox was found between endothelial cells and leukocytes. This finding is in agreement with a recent study using HUVECs by Görlich et al. Therefore, for gp91phox, no evidence for a structural basis of an altered substrate specificity could be detected. Analyses of the endothelial p67phox sequence revealed 2 bp substitutions with regard to the leukocyte sequence of this subunit. Because both bp substitutions are well-known polymorphisms of the NCF2 gene encoding the sequence of this subunit. It seems unlikely that they account for a general well-known polymorphisms of the NCF2 gene encoding the sequence of this subunit. Because both bp substitutions are well-known polymorphisms of the NCF2 gene encoding the sequence of this subunit. It seems unlikely that they account for a general difference in substrate specificity of the endothelial NAD(P)H oxidase. Therefore, we could not find a molecular basis at the DNA level for the altered substrate specificity in endothelial cells compared with leukocytes. Recently, several gp91phox-related proteins, including Mox-1 (now called Ncx-1), have been described in other cell types and tissues. These subunits could form another NAD(P)H oxidase complex as an alternative source of superoxide in HUVECs. This seems unlikely in HUVECs, however, because Mox-1 (Ncx-1) has been shown to be expressed in vascular smooth muscle cells but not in endothelial cells. Furthermore, our gp91phox primers do not amplify Mox-1, and we were unable to detect Mox-1 mRNA expression in our HUVECs either.

The rather low-output, constitutive endothelial superoxide release might be the consequence of a lower expression of NAD(P)H oxidase subunits in endothelial cells. This would result in a substantially lower number of NAD(P)H oxidase complexes in endothelial cells than in leukocytes. Therefore, we quantified the expression level of NAD(P)H oxidase subunits in leukocytes and endothelial cells by multistandard-assisted competitive RT-PCR. To correlate putative differences in subunit expression of leukocytes and endothelial cells with enzyme activity, DPI-inhibited superoxide anion formation was determined by the cytochrome c reduction assay in both cell types. The subunits p47phox and p22phox are expressed at a similar level in leukocytes and HUVECs. Striking differences between the 2 cell types were detected in the expression of p67phox and gp91phox. Both subunits are expressed at a much higher level in HUVECs. The most prominent difference was shown by gp91phox. Endothelial expression of this subunit corresponds to 1% of the expression in leukocytes. A similar difference between the 2 cell types was observed in the rate of NAD(P)H oxidase–dependent superoxide anion formation. Therefore, we conclude that the low rate of superoxide anion formation by the endothelial NAD(P)H oxidase is probably the consequence of the low expression of gp91phox in this cell type. Thus, the difference in kinetics of the superoxide release between leukocytes and endothelial cells might reflect a quantitative rather than a qualitative difference in NAD(P)H oxidase subunit expression in both cell types.

Using a common standard for all NAD(P)H oxidase subunits in competitive RT-PCR, we were able to compare the expression of subunits within 1 cell type. In HUVECs, gp91phox was expressed at the lowest level. Considering that each subunit has to be present once to establish an active NAD(P)H oxidase complex, gp91phox might therefore be referred to as the limiting subunit of NAD(P)H oxidase in endothelial cells. Our data suggest that the regulation of gp91phox expression might determine the number of enzyme complexes and hence the capacity for NAD(P)H oxidase–dependent superoxide anion formation in endothelial cells.

In further studies, the expression of limiting NAD(P)H oxidase subunit gp91phox and the superoxide release in response to oxLDL, a mediator of oxidative stress within the vessel wall, was determined in endothelial cells. In contrast to LDL, oxidatively modified LDL induces superoxide anion formation in cultured endothelial cells and intact aortic segments, and pharmacological evidence as well as experiments with antisense oligonucleotides hint at an NAD(P)H oxidase as the source for superoxide formation. In this study, we found a transient, dose-dependent induction of gp91phox expression in response to oxLDL in HUVECs. The transient character of gp91phox induction might be caused by a time-dependent depletion of oxLDL in the medium, because oxLDL can be rapidly taken up and degraded by endothelial cells via specific oxLDL receptors.

Furthermore, we were able to show that the oxLDL-mediated induction of NAD(P)H oxidase subunit gp91phox expression was accompanied by an induction of endothelial superoxide release within a similar time course. Therefore, we conclude that induction of gp91phox expression by the proatherosclerotic stimulus oxLDL might be the molecular basis for the observed elevation of superoxide release. These data further support our hypothesis that gp91phox expression determines the endothelial superoxide release.

Our data suggest a proatherosclerotic vicious circle involving endothelial gp91phox expression, superoxide release, and oxidative modification of LDL to oxLDL. Oxidative modification of LDL within the intima by a pathophysiologically elevated constitutive superoxide anion formation of vascular cells would result in an induction of gp91phox expression, with a subsequent elevation of endothelial superoxide release. This enhanced superoxide anion formation, in turn, would further promote the conversion of LDL to atherogenic oxLDL. Because it is known that elevated LDL plasma levels cause an enhanced influx of LDL into the intima, the suggested constitutive superoxide anion formation of vascular cells would lead to an enhanced oxidative modification of LDL. On the basis of this assumption, lipid-lowering therapy by treatment with HMG-CoA reductase inhibitors could result in a reduced oxLDL formation. As a consequence, a reduced vascular expression of gp91phox would be expected. We tested this hypothesis by determination of gp91phox mRNA expression in biopsies of internal mammary arteries of patients with or without HMG-CoA reductase inhibitor therapy undergoing aortocoronary bypass surgery. The observed downregulation of gp91phox expression by HMG-CoA reductase inhibitor therapy in human arteries of patients with coronary artery disease further supports our proposed model. Because the patients with HMG-CoA reductase inhibitor therapy had lower plasma levels of total cholesterol and LDL cholesterol, the reduced LDL plasma levels could mediate a reduced influx of LDL into the intima, which might subsequently cause a reduced formation of oxLDL. These reduced
oxLDL levels could at least in part explain the reduced gp91phox mRNA expression found in the internal mammary artery biopsies of our patients treated with statins. The lower gp91phox expression in the internal mammary arteries of the statin group was not due to reduced levels of phagocytic cells, because in the same biopsies, mRNA expression of the marker CD11c phagocytic cells was not different between the 2 groups of patients. Furthermore, HMG-CoA reductase inhibitors can reduce endothelial superoxide formation by preventing the isoprenylation of p21 Rac involved in the NAD(P)H oxidase activation.35

In summary, we show that oxLDL induces expression of gp91phox, the limiting subunit of endothelial NADPH oxidase, and superoxide anion formation in a dose-dependent manner in HUVECs. Our data support induction of proatherosclerotic oxidative stress in human endothelial cells in response to oxLDL and an antioxidative potential of HMG-CoA reductase inhibition via reduction of vascular NADPH oxidase expression.

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References
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