Nox4 as the Major Catalytic Component of an Endothelial NAD(P)H Oxidase

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Background—Recent evidence has suggested that reactive oxygen species are important signaling molecules in vascular cells and play a pivotal role in the development of vascular diseases. The activity of NAD(P)H oxidase has been identified as the major source of reactive oxygen species in vascular endothelial cells. However, the precise molecular structure and the mechanism of activation of the oxidase have remained poorly understood.

Methods and Results—Here, we investigated the molecular identities and the superoxide-producing activity of endothelial NAD(P)H oxidase. We found that Nox4, a homologue of gp91phox/Nox2, was abundantly expressed in endothelial cells. The expression of Nox4 in endothelial cells markedly exceeded that of other Nox proteins, including gp91phox/Nox2, and was affected by cell growth. Using electron spin resonance and chemiluminescence, we measured the superoxide production and found that the endothelial membranes had an NAD(P)H-dependent superoxide-producing activity comparable to that of the neutrophil membranes, whereas the activity was not enhanced by the 2 recombinant proteins p47phox and p67phox, in contrast to that of the neutrophil membranes. Downregulation of Nox4 by an antisense oligonucleotide reduced superoxide production in endothelial cells in vivo and in vitro.

Conclusions—These findings suggest that Nox4 may function as the major catalytic component of an endothelial NAD(P)H oxidase. (Circulation. 2004;109:227-233.)

Key Words: reactive oxygen species ■ NAD(P)H oxidase ■ endothelium

Vascular endothelial cells seem to play an important role in regulation of vascular tone and growth of vascular muscle cells. During several disease states, such as hypertension, diabetes, and hypercholesterolemia, endothelial function seems to be deteriorated. Such endothelial dysfunction may play a major role in the development of arteriosclerosis and cardiovascular disease. Reactive oxygen species (ROS), including hydrogen peroxide and superoxide, seem to be important signaling molecules in vascular cells. It has been shown that the activity of NAD(P)H oxidase, a superoxide-producing enzyme, is present in vascular endothelial, smooth muscle, and adventitial cells and contributes to the regulation of vascular tone and to cardinal steps of the development of arteriosclerosis, ie, endothelial dysfunction, abnormal smooth muscle cell growth, and inflammation.

The phagocyte NADPH oxidase is composed of 2 essential membrane-bound components, gp91phox/Nox2 and p22phox, which compose flavocytochrome b55d, and 4 cytosolic components, p47phox, p67phox, p40phox, and the small G protein Rac1/2. Upon stimulation, the 4 cytosolic proteins translocate to the membrane, assemble with the cytochrome b55d, and thereby increase the activity of NADPH oxidase. The activated oxidase produces a large amount of superoxide and plays a pivotal role in host defense against microbial infection.

Conversely, the precise structure and the mechanisms of activation of the NAD(P)H oxidase in vascular endothelial cells are still poorly understood. Although previous studies have confirmed that all of the oxidase components identical to the phagocyte NADPH oxidase are present in endothelial cells, the endothelial NAD(P)H oxidase is considered to be different from the phagocyte oxidase in that the endothelial oxidase is continuously active at a low level even in the unstimulated conditions and the oxidase does not generate as high levels of ROS as the burst activity of the phagocytic enzyme. Li and Shah recently showed that the endothelial oxidase, in contrast to the phagocyte oxidase, exists as a preassembled intracellular complex associated with the cytoskeleton. Recently, 4 homologues of gp91phox/Nox2, called Nox1 and Nox3 to 5, have been identified in nonphagocytic cells, and the simultaneous presence of multiple Nox proteins was demonstrated in one cell type, raising the possibility that endothelial cells may also express another Nox protein.
In the present study, using real-time polymerase chain reaction (PCR), we examined the expression level of all the components of the oxidase, including 5 Nox proteins (Nox1–5), in vascular endothelial cells. Furthermore, we investigated the superoxide-producing activity of the endothelial membranes using chemiluminescence and electron spin resonance (ESR).

**Methods**

**Culture of Endothelial Cells**

Rat aortic endothelial cells (RAECs) were collected from the aorta of male Sprague-Dawley rats (KYUDO Co Ltd, Tosu, Japan) 4 to 6 weeks old as described previously and cultured with DMEM supplemented with 10% FBS (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and cultured with 10% FBS (Invitrogen). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics and cultured with 10% FBS (Invitrogen).

**Preparation of Neutrophils**

Human neutrophils were isolated from venous blood of healthy volunteers by dextran sedimentation, hypotonic exposure, and the Ficoll-Paque method (Amersham Pharmacia Biotech) as described previously.

**Reverse Transcription–PCR**

Total RNA was prepared with TRizol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed by AMV transcriptase (Roche) in a total volume of 20 μL. Using 0.5 μL of the product as a template, PCR was performed with gene-specific primers as shown in Table 1.

**Quantitative Real-Time PCR**

The reverse transcriptase (RT) products (0.5 μL) were amplified with LightCycler (Roche) in the reaction mixture (20 μL) containing 2 μL of LightCycler-FastStart DNA Master SYBR Green I, 0.5 μmol/L of each primer, and 3 mmol/L MgCl₂. The mRNA levels were determined by quantitative real-time PCR performed twice for each of the independently prepared total RNAs. The numbers were standardized by those of 2 housekeeping genes, GAPDH and cyclophilin B.

**Plasmid Construction and Preparation of Recombinant Proteins**

Plasmid constructions (pGEX-2T–p47phox, pGEX-2T–p67phox-N) were performed as described previously. Proteins fused to GST were expressed in _Escherichia coli_ strain BL21 and purified by glutathione–Sepharose-4B (Amersham Pharmacia Biotech).

**Superoxide Measurement by Chemiluminescence**

Superoxide was measured by chemiluminescence with lucigenin or 50 μL Diogenes. After incubation of membranes for 1 minute with or without the recombinant proteins GST–p47phox, GST–p67phox-N, and GST–Rac2 (Q61L), in the presence of 100 μmol/L SDS at room temperature, 0.5 μmol/L NADPH was added to the reaction mixture. The chemiluminescence was measured with a luminometer (MiniLMat LB9506; EG&G Berthold). The reaction was stopped by addition of superoxide dismutase (SOD; 200 U/mL).

**Superoxide Measurement by ESR**

ESR measurement was performed at room temperature with a JES-RE1X ESR spectrometer (JEOL). The setting conditions of the instrument were as follows: Magnetic field was 338.6 ± 7.5 mT, modulation amplitude 0.1 mT, time constant 300 ms, modulation frequency 9.425 GHz, and sweep time 8 minutes. Membrane protein (20 μg) was incubated with 10 mmol/L 5-(diethoxyphosphoryl)-5-methyl-1-pyrrroline N-oxide (DEPMPO) in 100 mmol/L potassium phosphate, pH 7.0, containing 5 μmol/L FAD, 1 mmol/L MgCl₂, 0.1 mmol/L NaCl, and 5 μmol/L lucigenin or 50 μL Diogenes. After incubation of membranes for 1 minute with or without the recombinant proteins GST–p47phox, GST–p67phox-N, and GST–Rac2 (Q61L), in the presence of 100 μmol/L SDS at room temperature, 0.5 μmol/L NADPH was added to the reaction mixture. The chemiluminescence was measured with a luminometer (MiniLMat LB9506; EG&G Berthold). The reaction was stopped by addition of superoxide dismutase (SOD; 200 U/mL).

**Table 1. Nucleotide Sequences of Primers Used for RT-PCR**

<table>
<thead>
<tr>
<th>Primer Code</th>
<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r p22phox*</td>
<td>TGGCCCTGATCTCCTACGACAG</td>
<td>AGGCCACGGAACGAGTAAGT</td>
</tr>
<tr>
<td>h p22phox</td>
<td>GTACCTTGGTCGTACCTCCTCA</td>
<td>CGGCCCGAACATAGTATTCC</td>
</tr>
<tr>
<td>r and h Nox1*</td>
<td>CTTCCTCACTGGCTGGGATA</td>
<td>TGGACAGATTTCGCCAGGCT</td>
</tr>
<tr>
<td>r and h gp91phox/Nox2</td>
<td>CCAATGGAATGTTCCAGCTT</td>
<td>GCACAGCACTGAAAGTAGAT</td>
</tr>
<tr>
<td>r and h gp91phox/Nox2</td>
<td>ATGGGGAATGCTGGTGTG</td>
<td>GTTAAGAAGCTTCTGTTGAA</td>
</tr>
<tr>
<td>h gp91phox/Nox2</td>
<td>TGTCCAAGCTGGAGTGCTGTA</td>
<td>TTACGGCAATGAGCTTTCA</td>
</tr>
<tr>
<td>r and h Nox3</td>
<td>AGTCCAACAGATGGGATA</td>
<td>CTGGCCACATAGGTGTT</td>
</tr>
<tr>
<td>r and h Nox4*</td>
<td>AGCAATCTGGCCAGGCT</td>
<td>CCAGCAATGAGCTTTCA</td>
</tr>
<tr>
<td>r and h Nox5</td>
<td>AAGCATCTGGCCAGGCT</td>
<td>CCAGCAATGAGCTTTCA</td>
</tr>
<tr>
<td>r p47phox*</td>
<td>TCACCGGATCTAGGATTTC</td>
<td>CCCATAGGCGTGTGAA</td>
</tr>
<tr>
<td>h p47phox</td>
<td>TTGAGAAGCGCTTGTACCT</td>
<td>TGTCACACCACCTGAGGCT</td>
</tr>
<tr>
<td>r and h p67phox*</td>
<td>CAGTCCACCTGGTGTCCT</td>
<td>TCTTGGCCACGCTGAGGCT</td>
</tr>
<tr>
<td>r and h GAPDH*</td>
<td>TGACCGGGAGGCTCAGTG</td>
<td>TCCACACCCCTTGGGTGTA</td>
</tr>
<tr>
<td>r and h cyclophilin B*</td>
<td>ATGGCCACAGAGGAAAGGCC</td>
<td>ATGGATCACATCTCTAGGG</td>
</tr>
</tbody>
</table>

*r and h indicate rat and human, respectively.

Primers used for real-time PCR.
Antisense Transfection
Phosphorothioate oligonucleotides, including Nox4 antisense (5’-GCGACAGCCATGCGGCC-3’), sense (5’-GGCGGCATGG-CCTGTTCC-3’), scramble (5’-GGGTAGGTCTCATCCTAGG-3’), previously reported23 p22phox antisense (5’-GATCTGCCCCAT-GTGTGC-3’), and p22phox sense (5’-GGTCTTCACCA-TGGGGCAGATC-3’), were prepared (Amersham-Pharmacia Biotech). Indicated concentrations of oligonucleotide and 1 μg of lipofectin (Invitrogen) per 1 μl oligonucleotide were diluted with Opti-MEM (Invitrogen) and incubated for 30 minutes, respectively. Then, the oligonucleotide and the lipofectin solutions were mixed and incubated for 15 minutes. The endothelial cells with 60% to 70% confluence were treated with the oligonucleotide/lipofectin mixture for 4 hours. After removing the mixture, the cells were incubated in DMEM with 10% FBS for 30 hours before the following experiments.

Superoxide Detection by Fluorescence Microscopy
The oxidative fluorescent dye dihydroethidium was used to evaluate superoxide production in vivo as described previously.26 The oxidative fluorescent dye dihydroethidium was used to evaluate superoxide production in vivo as described previously.26 The endothelial cells on 35-mm glass-bottom dishes (MatTek) were treated with the oligonucleotide/lipofectin mixture for 4 hours. After removing the mixture, the cells were incubated in DMEM with 10% FBS for 30 hours before the following experiments.

Statistical Analysis
Values were expressed as mean±SEM. Results were compared by unpaired t test or 1-way factorial ANOVA followed by a post hoc Scheffe’s comparison test. A value of P<0.05 was accepted as significant.

Results
Components of Oxidase in Endothelial Cells
Neutrophils highly expressed 2 membrane components, gp91phox/Nox2 and p22phox, and the specific cytosolic components p47phox and p67phox (Figure 1). The endothelial cells also expressed all of the 4 components; however, the expression levels of gp91phox/Nox2 and p47phox in both RAECs and HUVECs and that of p67phox in HUVECs were very limited (Figure 1). We used real-time PCR to quantify the expression number of each component. The real-time PCR disclosed that the expression numbers of gp91phox/Nox2 and p47phox were considerably lower than those of neutrophils (Table 2).

Because the expression level of gp91phox/Nox2 was quite low in the endothelial cells, we examined the expression of other Nox proteins (Nox1 and Nox3–5) in RAECs and HUVECs. Among the Nox proteins, Nox4 was highly expressed in the endothelial cells (Figure 2A). The expression number of Nox4 in the endothelial cells was estimated to be ≥10 000 copies per 25 mg of total RNA at a proliferating state by real-time PCR (Table 2). Expression of Nox1 was also detected in the endothelial cells, but the expression level was significantly lower (Figure 2A).27

Regulated Expression of Nox4 in Endothelial Cells
We examined whether the Nox4 expression was affected by culture conditions in the endothelial cells. When FBS was removed from culture medium for 24 hours, Nox4 expression was increased by 2– to 3-fold. Conversely, readdition of FBS suppressed the Nox4 expression to the basal level in 12 hours (Figure 2B). The change of p22phox expression after the removal and readdition of serum was almost in accordance with that of Nox4 (Figure 2C).

Superoxide-Producing Activity of Endothelial Membranes
Because Nox4 protein is speculated to exist in membranes by its structural similarity to gp91phox/Nox2,19,20 we investigated whether the membrane fraction of RAECs was capable of producing superoxide. Addition of NADPH to the membrane fraction of RAECs promptly increased the chemiluminescence using a low concentration of lucigenin16,18; however, SOD did not abolish the signal16 (data not shown). Conversely, an NADPH-induced increase of chemiluminescence by use of Diogenes was completely inhibited by SOD (Figure 3A), indicating that the Diogenes is more reliable for detection of superoxide. In this method, the superoxide-producing activity of RAEC membranes was almost equivalent to that of neutrophil membranes (data not shown).

Table 2. Quantification of mRNA of the NADPH Oxidase Components by Real-Time PCR

<table>
<thead>
<tr>
<th></th>
<th>gp91phox/Nox2</th>
<th>p22phox</th>
<th>p47phox</th>
<th>p67phox</th>
<th>Nox4</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAECs</td>
<td>6.2±3.1</td>
<td>12.779.3±1740.2</td>
<td>2.5±0.5</td>
<td>2539.5±724.7</td>
<td>14 666.5±6975.6</td>
</tr>
<tr>
<td>HUVECs</td>
<td>3.3±1.7</td>
<td>877.8±197.9</td>
<td>2.5±0.9</td>
<td>41.8±13.5</td>
<td>16 961.3±2499.9</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>191 117.0±60 046.5</td>
<td>81 082.5±14 508.1</td>
<td>151 639.2±54 698.9</td>
<td>76 299.3±16 960.6</td>
<td>10.2±3.5</td>
</tr>
</tbody>
</table>

RNA was prepared from the indicated cells, reverse-transcribed, and quantified by real-time PCR. Values (mean±SEM) are expressed as copy numbers (n=4), which are standardized by 2 housekeeping genes, GAPDH and cyclophilin B.
NADPH-induced increases of chemiluminescence in both RAEC and neutrophil membranes were inhibited by 10 μmol/L diphenylene iodonium but not by 100 μmol/L N\textsuperscript{G}-nitro-L-arginine methyl ester, 100 μmol/L indomethacin, and 100 μmol/L oxypurinol (data not shown). Furthermore, we used ESR to show that the RAEC membranes produced both superoxide (DEPMPO-OOH adduct) and hydroxyl radical (DEPMPO-OH adduct) in a NADPH-dependent manner (Figure 3B). Addition of SOD (Figure 3B) but not catalase (data not shown) completely inhibited both signals, indicating that hydroxyl radical was derived from superoxide.\textsuperscript{28} The total amount of superoxide and hydroxyl radical produced by RAEC membranes was almost equivalent to that of the unstimulated neutrophil membranes (Figures 3B, 3C, and 4C). The effects of NADH on the activity of the endothelial NAD(P)H oxidase were almost similar to those of NADPH, and the effect of each substrate was dependent on its concentration (data not shown).
Role of the Cytosolic Components p47phox and p67phox in Endothelial Cells

To examine the activity of the cytosolic factors, we prepared 3 recombinant proteins, GST–p47phox, GST–p67phox, and GST–Rac2. When the recombinant proteins were added to the neutrophil membranes, the typical DEPMPO-OOH adduct was readily detected and was estimated to be 10-fold higher than that of the membranes alone (Figure 4, B and C). When the recombinant proteins were added to RAEC membranes, ESR signals were almost similar to those of RAEC membranes alone (Figure 4, A and C). We obtained similar results by the cell-free system using Diogenes (data not shown).

Nox4 Is Involved in Superoxide Production in Endothelial Cells

To elucidate whether Nox4 is involved in the endothelial superoxide production, we used a Nox4 antisense oligonucleotide. The Nox4 antisense oligonucleotide significantly suppressed Nox4 expression in a concentration-dependent manner (Figure 5A) but did not affect the expression of gp91phox/Nox2 at all (data not shown). Using fluorescent microscopy, we confirmed that intracellular superoxide production was decreased in accordance with antisense oligonucleotide–dependent suppression of Nox4 (Figure 5B). Furthermore, to quantify the reduction of superoxide production by the oligonucleotides, we performed the chemiluminescence experiment using membrane fractions treated with the oligonucleotides. The superoxide-producing activity of the membrane fractions treated with the Nox4 antisense oligonucleotide was significantly smaller than that treated with sense or scramble oligonucleotide (Figure 5C). The degree of reduction in superoxide production by the Nox4 antisense (≈50%) was coincident with the inhibitory effect on the Nox4 mRNA by the Nox4 antisense. A p22phox antisense oligonucleotide similarly reduced the superoxide production of the endothelial membranes by 40% to 50% (Figure 5C).

Discussion

Here, we demonstrated a new finding that Nox4 may function as the major catalytic component of an endothelial NAD(P)H oxidase.

Presence of Nox4 in Endothelial Cells

In the present study, we have confirmed, using RT-PCR, that Nox4 is abundantly expressed in both RAECs and HUVECs. The expression level determined by real-time PCR was comparable to that of vascular muscle cells, which had already been established as Nox4-expressing cells.23 Moreover, the activity of the endothelial NAD(P)H oxidase was similar with Nox4-containing oxidase.20 The endothelial membranes produced an amount of superoxide comparable to that of the unstimulated neutrophil membranes. However, the oxidase activity of the endothelial membranes was not enhanced significantly by the cytosolic proteins p47phox and p67phox.20 The NAD(P)H-dependent superoxide production in the endothelial cells was inhibited by diphenylene iodonium, an inhibitor of flavoproteins, but not by other inhibitors, such as Nω-nitro-arginine methyl ester, indomethacin, and oxypurinol, suggesting that the superoxide production was derived from Nox family proteins. We have also shown that Nox4 suppression by a Nox4 antisense oligonucleotide is coincident with the reduction of the superoxide-producing activity of the endothelial membranes. In addition, the effect of a p22phox antisense oligonucleotide on superoxide production in endothelial membranes was comparable to that of the Nox4 antisense. These findings suggest that Nox4 may be
present and functionally active with p22phox as a superoxide-producing enzyme in the endothelial cells. Sorescu et al\textsuperscript{27} also recently reported that Nox4 is abundantly expressed in cultured endothelial cells. We also cannot exclude the possibility that the residual activity is derived from other superoxide-producing enzymes, such as gp91phox/Nox2, as described below.

**Presence of Multiple Nox Proteins in Endothelial Cells**

It is well established that gp91phox/Nox2 is expressed in endothelial cells.\textsuperscript{17,18} In the present study, we also found that a small but significant amount of gp91phox/Nox2 is expressed in both RAECs and HUVECs. It was reported that Nox1 and Nox4 are coexpressed in vascular muscle cells.\textsuperscript{23} Thus, Nox4 as well as gp91phox/Nox2 may function as a superoxide-producing enzyme in the endothelial cells. Although all Nox proteins (Nox1–5) are considered to be involved in superoxide production,\textsuperscript{10,19,20,22} it remains poorly understood how cells deal with distinct Nox proteins. A possible explanation may be that intracellular localization of each Nox protein is different, which may determine the role of each Nox protein.\textsuperscript{19,23} gp91phox/Nox2 is established to exist in the plasma or granular membranes of neutrophils. By use of the PSORT program, it is predicted that Nox4 may be localized in endoplasmic reticulum.\textsuperscript{23} Thus, it may be possible that Nox4 and gp91phox/Nox2 are expressed in the distinct membrane of the endothelial cells and have different roles in the cell functions.

It has been reported that gp91phox/Nox2 and p22phox stabilize each other at the protein level.\textsuperscript{12} Because the expression of p22phox was much higher than that of gp91phox/Nox2 in endothelial cells,\textsuperscript{17} one might anticipate that p22phox could not exist stably at the protein level. Lassegue et al\textsuperscript{23} suggested that both Nox1 and Nox4 may form complexes with p22phox and compose functional cytochromes in vascular muscle cells. Thus, it may be possible that Nox4 as well as gp91phox/Nox2 may form a complex with p22phox in endothelial cells.

**Figure 5.** Effects of a Nox4 antisense oligonucleotide on Nox4 expression and superoxide production in RAECs. A, Indicated oligonucleotides were transduced into RAECs. Total RNA was prepared 30 hours later, and real-time PCR was performed. Values are mean±SEM (n=4; *P<0.05 vs control). B, Effects of each 0.5 μmol/L oligonucleotide on superoxide production in unstimulated RAECs were determined by fluorescence microscopy using dihydroethidium. C, Membrane fractions were prepared 30 hours after treatment of endothelial cells with indicated oligonucleotide (0.5 μmol/L). Total chemiluminescence changes induced by 0.5 mmol/L NADPH for 5 minutes were expressed. Values are mean±SEM (n=8; *P<0.05 vs scramble).
Role of Nox4 in Endothelial Cells

Nox4 was originally identified from kidney, where Nox4 probably serves as an oxygen sensor to regulate the production of erythropoietin.\(^\text{19,20}\) Thereafter, it was disclosed that Nox4 functions as an important membrane component of endothelial cells.\(^\text{21,23}\) In atherosclerotic lesions,\(^\text{27,29}\) Nox4 was highly expressed with Nox1 and p22phox and produced superoxide, suggesting that Nox4 may play some important role in formation of atherosclerotic lesions. Furthermore, it has been reported that Nox4 expression is decreased by proliferative stimuli, such as angiotensin II, platelet-derived growth factor, and serum, in cultured vascular muscle cells.\(^\text{23}\) In the present study, the Nox4 expression in endothelial cells was significantly increased with the suppression of cell proliferation by serum removal and decreased by addition of serum. Taken together with the previous observation that overexpression of Nox4 in fibroblast NIH 3T3 cells decreased the rate of proliferation of the cells,\(^\text{19,20}\) it is possible that Nox4 plays a role in the regulation of cell growth or cell survival in endothelial cells.

In conclusion, we provide here several lines of evidence that Nox4 functions as an important membrane component of an endothelial NAD(P)H oxidase along with the oxidase consisting of gp91phox/Nox2.

References

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