Role of Increased Production of Superoxide Anions by NAD(P)H Oxidase and Xanthine Oxidase in Prolonged Endotoxemia

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Abstract—Superoxide anions (O$_2^-$) are supposedly involved in the pathogenesis of endothelial dysfunction. We investigated whether the enhanced formation of O$_2^-$ is involved in the attenuation of endothelium-dependent relaxation induced by lipopolysaccharide (LPS). Rats were injected with LPS (10 mg/kg IP), the aorta was removed after 12 or 30 hours, and generation of O$_2^-$, H$_2$O$_2$, and ONOO$^-$ was measured using chemiluminescence assays. Protein tyrosine nitration and expression of xanthine oxidase (XO), NAD(P)H oxidase, and manganese superoxide dismutase were determined by Western or Northern blotting, and endothelium-dependent relaxation in aortic rings was studied. LPS treatment increased vascular O$_2^-$ (from 35±2 cpm/ring at baseline to 166±21 cpm/ring at 12 hours and 225±16 cpm/ring at 30 hours) and H$_2$O$_2$ formation, which was partially sensitive to the NAD(P)H oxidase inhibitor diphenylene iodonium at both time points studied and to the XO inhibitor oxyypurinol only 30 hours after LPS treatment. Expression of XO and NAD(P)H oxidase (p22phox, p67phox, and gp91phox) were increased by LPS in a time-dependent manner, as were protein tyrosine nitration and ONOO$^-$ formation. LPS also induced expression of the oxidative stress–sensitive protein manganese superoxide dismutase. Endothelium-dependent relaxation was impaired after LPS treatment and could not be restored by inhibition of inducible NO synthase. Inhibition of O$_2^-$ with superoxide dismutase, oxyypurinol, tiron, or the superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin chloride did not restore but further deteriorated the relaxation of LPS-treated rings. In summary, treatment of rats with LPS enhances vascular expression of XO and NAD(P)H oxidase and increases formation of O$_2^-$ and ONOO$^-$. Because removal of O$_2^-$ compromised rather than restored endothelium-dependent relaxation, a direct role of O$_2^-$ in the induction of endothelial dysfunction is unlikely. Other mechanisms, such as prolonged protein tyrosine nitration by peroxynitrite (which is formed from NO and O$_2^-$) or downregulation of the NO effector pathway, are more likely to be involved. (Hypertension. 1999;33:1243-1249.)

Key Words: superoxide dismutase ■ endothelium ■ lipopolysaccharides ■ nitric oxide ■ xanthine oxidase ■ NAD(P)H oxidase

Endothelial dysfunction is a phenomenon characterized by impaired endothelium-dependent relaxation and has been shown to be related to increased scavenging of NO by O$_2^-$ rather than impaired generation of NO in various disease models. The sources of O$_2^-$ within the vessel wall have not been fully identified, but evidence suggests that increased generation of O$_2^-$ is at least partially due to activation of vascular NAD(P)H oxidase and xanthine oxidase (XO). Severe hypotension and hyporesponsiveness to vasoconstrictors and endothelium-dependent dilator agents are hallmarks of sepsis, eg, after exposure to bacterial lipopolysaccharide (LPS). Although LPS-induced vascular hyporesponsiveness to constric- tor agents may be partially due to excessive generation of NO by inducible NO synthase (iNOS), the mechanism underlying LPS-induced endothelial dysfunction is not clear. We investigated whether enhanced formation of O$_2^-$ could be involved in the development of endothelial dysfunction after exposure to LPS.

Methods

Materials

Diphenylene iodonium (DPI), U44069, and aminohydromethylhia- zine (AMT) were obtained from Alexis. Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from Calbiochem. Recombinant human superoxide dismutase (rhSOD) was a gift from Grünenthal Inc. Rat vascular smooth muscle cell p22phox cDNA was a kind gift from K.K. Griendling, Emory University. XO cDNA was provided by M. Saksela, University of Helsinki. The mouse

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monoclonal nitrotyrosine antibody was a gift from J.S. Beckman, University of Alabama. Anti-leukocyte NADPH oxidase antibodies were generously provided by M.T. Quinn, Montana State University; F. Wientjes, University College London; and O.T.G. Jones, University of Bristol. The anti-rat XO antibody was kindly donated by T. Nishino, Nippon Medical School. The anti-iNOS antibody was from J. Pfeilschifter, University of Frankfurt. Anti-manganese superoxide dismutase (MnSOD) and MnSOD cDNA were prepared as previously described. All antibodies were made against human enzymes and were from rabbit unless otherwise stated.

Animals and Study Protocol
The study was approved by the Hannover and Frankfurt district government. One hundred-two male Sprague-Dawley rats (250 to 300 g) were assigned to receive LPS (Escherichia coli LPS serotype 011:B4, 10 mg/kg IP) or placebo (normal saline). The chosen dose of LPS resulted in a mortality rate of <5% within 30 hours after injection. Rats were killed after 12 or 30 hours, and the aorta was aorta removed and cleaned. The cleaned aorta was partially cut into rings 3 mm in length for organ chamber studies and radical detection, and the remainder was snap-frozen in liquid nitrogen and stored at −86°C.

Measurements of Reactive Oxygen Species and Peroxynitrite
Measurements of reactive oxygen species in intact aortic rings were performed using chemiluminescence assays as previously described. The contribution of vascular NAD(P)H oxidase, XO, or endothelium to O₂⁻ formation was assessed by measuring the sensitivity of the chemiluminescence signal to DPI (10 μmol/L), oxypurinol (1 mmol/L), or denudation of the ring, respectively. Luminol (600 μmol/L) was used to assess peroxynitrite (ONOO⁻) production as previously described. H₂O₂ was determined by use of a peroxidase-based chemiluminescence assay as described elsewhere.

Immunoblotting
Frozen aortic tissue was homogenized and suspended in Tris buffer (50 mmol/L, pH 7.4) containing EGTA (1 mmol/L), PMSF (44 μmol/L), trypsin inhibitor (10 μg/mL), and leupeptin (2 μg/mL). After centrifugation at 29 000 g for 20 minutes at 4°C, the supernatant containing the cytoplasmic fraction was removed and used for immunoblotting of p47phox, p22phox, and gp91phox. For the anti-nitrotyrosine Western blot, 1000-g supernatants of crude homogenates were used. Fifty micrograms of protein was subjected to SDS–polyacrylamide gel electrophoresis as previously described. Human leukocytes were used as positive controls for the NAD(P)H oxidase subunits proteins. Proteins were detected using their respective antibodies, which were linked with the appropriate horseradish peroxidase–coupled secondary antibody (Calbiochem), and were visualized by enhanced chemiluminescence (Amersham).

Northern Blot Analysis
Tissue was homogenized in guanidine-thiocyanate buffer. RNA was extracted according to the method of Chomczynski and Sacchi. RNA (20 μg/lane) was separated on 1% formaldehyde-agarose gels. Identical loading of the gel lanes was confirmed by comparing the ethidium bromide staining of the 18S bands. Blotting, cross-linking, and hybridization were performed with cDNA for MnSOD, XO, and p22phox, respectively, as described earlier.

Determination of XO Activity
XO activity was determined in the cytoplasmic fraction of rat aortae using a chemiluminescence assay in which buttermilk XO served as standard. Samples (50 μg) were preincubated in 0.1 M of Tris-HCl and 1 mmol/L of EDTA, pH 9.0, containing 230 μmol/L of lucigenin. After the background signal was recorded, the reaction was started with 50 μmol/L of xanthine. Once a stable plateau was reached, the chemiluminescence signal was recorded over a 2-minute period. Oxypurinol (1 mmol/L) completely inhibited the signal but had no effect on background chemiluminescence.

Organ Chamber Experiments
Aortic rings were connected to isometric force transducers in organ chambers as described earlier using a passive tension of 2g. Rings were contracted to 50% of maximal tension with U44069. To assess endothelium-dependent and -independent relaxation, atacetycholine (0.001 to 10 μmol/L) or sodium nitroprusside (SNP, 0.0001 to 1 μmol/L) was cumulatively added to the organ chambers. In some experiments, iNOS was inhibited by adding the iNOS inhibitor AMT (1 μmol/L) to the solution. rhSOD (1 μmol/L), the O₂⁻ scavenger tiron (10 mmol/L), and MnTBAP (50 μmol/L) were used to study the effect of inhibition of vascular O₂⁻ production. Oxypurinol (1 mmol/L) was used to inhibit XO. All substances were added to the bath solution 45 minutes before dose-response curves were determined. Oxypurinol, MnTBAP, and tiron were washed out immediately before concentration-response curves were obtained.

Statistical Analysis
Values are mean±SEM and were compared by ANOVA for repeated measurements, followed by the Newman-Keuls test. Densitometric analysis of blots was performed using the PC version of NIH-Image from Scion Corp.

Results
Vascular O₂⁻ and H₂O₂ Generation
In aortic segments removed 12 hours after LPS injection, vascular O₂⁻ generation, as measured by lucigenin chemiluminescence, was increased by 4.7-fold and was further increased in segments removed 30 hours after LPS exposure (Figure 1A). Vascular H₂O₂ formation, as measured by horseradish peroxidase–luminol–coupled chemiluminescence, was increased by 1.9-fold in segments removed 30 hours after LPS treatment as compared with controls (Figure 1B). Addition of catalase (200 U/mL) completely abolished the H₂O₂ signal, confirming the specificity of the assay (data not shown).

 Twelve hours after LPS injection, the vascular formation of O₂⁻, as measured by lucigenin chemiluminescence, was decreased by the NAD(P)H oxidase inhibitor DPI but not by the XO inhibitor oxypurinol. DPI and oxypurinol both reduced O₂⁻ formation in segments removed 30 hours after LPS treatment. Denudation of rings impaired the signal by only 20% in controls but by 65% and 35% in rings obtained 12 and 30 hours after LPS treatment, respectively (Figure 1C).

Effect of LPS Treatment on XO and Vascular NAD(P)H Oxidase
XO/xanthine dehydrogenase (XDH) mRNA was detected in aortic tissue from LPS-treated rats but not in control rats. Western blot analysis revealed a time-dependent increase in expression of LPS, accompanied by an increase in XO activity (Figure 2).

LPS induced a time-dependent increase in expression of NAD(P)H oxidase components gp91phox and p67phox. For the anti-nitrotyrosine Western blot, 1000-g supernatants of crude homogenates were used. Fifty micrograms of protein was subjected to SDS–polyacrylamide gel electrophoresis as previously described. Human leukocytes were used as positive controls for the NAD(P)H oxidase subunits proteins. Proteins were detected using their respective antibodies, which were linked with the appropriate horseradish peroxidase–coupled secondary antibody (Calbiochem), and were visualized by enhanced chemiluminescence (Amersham).
analysis using cDNA directed against rat vascular smooth muscle cell p22phox revealed marked upregulation of p22phox mRNA by LPS (Figure 3).

Expression of MnSOD

Expression of MnSOD is reportedly controlled by redox-sensitive mechanisms. It is therefore conceivable that the oxidative stress induced by LPS treatment may affect Mn-SOD expression. Indeed, MnSOD mRNA and protein were markedly upregulated in rat aortic tissue by LPS treatment. mRNA expression was maximal 12 hours after LPS injection, whereas protein expression increased for up to 30 hours (Figure 4).

Vascular iNOS Induction and Peroxynitrite Generation

iNOS protein was determined in aortic segments removed 12 or 30 hours after LPS injection. Protein expression was maximal after 12 hours and markedly reduced but still detectable after 30 hours. In parallel experiments using luminol chemiluminescence, a 7.2-fold increase in ONOO− generation was measured 30 hours after LPS injection. In accordance with this observation, nitration of protein tyrosine residues was increased in a time-dependent manner after LPS treatment (Figure 5).

Vascular Relaxation After LPS Treatment

Maximal contractions induced by KCl and endothelium-dependent relaxation in response to acetylcholine were significantly impaired in aortic rings from LPS-treated animals (25 ± 0.7 versus 16 ± 0.9 and 16 ± 0.7 N, P < 0.05). Relaxations elicited by the NO donor SNP were impaired in a time-dependent manner in aortic rings from LPS-treated rats (98 ± 1% versus 89 ± 3% and 82 ± 3%, P < 0.05) and were not restored by removing the endothelium (data not shown). Acetylcholine-induced relaxation was also impaired in a time-dependent manner. Addition of rhSOD to preconstricted rings resulted in marked relaxation that was more pronounced in rings from control rats than in rings from LPS-treated rats (Figure 6A). rhSOD failed to improve acetylcholine-induced relaxation at any time point (Figure 6B and 6C) but impaired relaxation in aortic rings obtained from rats 30 hours after LPS treatment (Figure 6C). Tiron and MnTBAP had no effect on relaxant responses to acetylcholine in aortic rings from

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Figure 1. Formation of reactive oxygen species from rats treated with LPS (10 mg/kg IP). A, O2− formation in aortic tissue from control and treated rats 12 and 30 hours after LPS injection. B, H2O2 formation in aortic tissues from control and treated rats 30 hours after LPS injection. The chemiluminescence signals in A and B are expressed in counts per minute per aortic ring (CPM). C, Effect of endothelial denudation, DPI (10 μmol/L), and oxypurinol (1 mmol/L) on O2− production. Aortic tissue was obtained from control rats ([]) and LPS-treated rats ([]) and 30 hours ([]) after injection. *P < 0.05 vs control. #P < 0.05 vs 30 hours after LPS injection.

![Figure 2](http://hyper.ahajournals.org/)

Figure 2. Effect of LPS (10 mg/kg IP) on XO expression and activity. Aortic tissue samples were obtained from control rats and from rats 12 and 30 hours after LPS injection. Top, XO/XDH mRNA expression as detected by Northern blot analysis. Middle, XO/XDH protein expression as detected by Western blot analysis using a specific polyclonal antibody. The band at ~130 kDa reflects the native enzyme, whereas the other bands are proteolytically cleaved enzyme fragments that have XO but not XDH activity. Bottom, XO activity as measured by chemiluminescence in homogenates. Numbers below blots show results of densitometric analysis. *P < 0.05.

![Figure 3](http://hyper.ahajournals.org/)

Figure 3. Effect of LPS treatment on expression of components of the leukocyte-type NAD(P)H oxidase in rat aortic tissue. Northern blot analysis showed the effect of LPS on expression of rat vascular type p22phox mRNA, and Western blot analysis showed the expression of p67phox and gp91phox using antibodies directed against the human leukocyte-type NAD(P)H oxidase. *P < 0.05.
control rats but slightly impaired relaxation in rings from rats 30 hours after LPS injection. Similar results were obtained with oxypurinol. Incubation of aortic rings with AMT resulted in similar impairment of relaxation in the control and 30-hour groups (Figure 7).

Discussion

In the present study, we demonstrated that a single application of LPS to rats in vivo increases aortic $O_2^-$ formation for up to 30 hours by the induction of XO and the "vascular-type" NAD(P)H oxidase. In addition, we observed a concomitant increase in MnSOD expression as well as ONOO$^-$ and nitrotyrosine formation. LPS treatment impaired acetylcholine-induced relaxation. This effect was not mediated directly by $O_2^-$, and rather than being beneficial, the scavenging of $O_2^-$ deteriorated relaxation.

A substantial portion of LPS-induced $O_2^-$ generation was sensitive to denudation of the rings. Nevertheless, whether this could be attributed to direct endothelial $O_2^-$ generation cannot be determined by the present study. Glycocalyx-bound enzymes and inflammatory cells adhering to the vascular lumen are also removed by the denudation process and have been shown to mimic endothelial $O_2^-$ generation in the lucigenin assay.

Moreover, the suitability of the lucigenin chemiluminescence method used here to determine $O_2^-$ generation, especially in endothelial cells, was recently questioned. Therefore, $H_2O_2$ generation was also measured using horseradish peroxidase-luminol–coupled chemiluminescence and confirmed increased radical generation 30 hours after LPS treatment.

Although vascular cells can acutely produce $O_2^-$ on exposure to LPS, the prolonged generation of $O_2^-$ requires induction of $O_2^-$-generating enzymes. There are a number of possible enzymatic sources of $O_2^-$ in the vascular wall. The observation that the LPS-induced $O_2^-$ formation in our model was partially sensitive to DPI is suggestive of the involvement of flavine-dependent enzymes, such as XO, NOS, and NAD(P)H oxidase. In the present study, we observed marked oxypurinol-sensitive chemiluminescence in the rat aorta 30 hours after injection of LPS; this was paralleled by a significant increase in XO activity and mRNA and protein expression. Moreover, Western blot analysis revealed lower-molecular-weight bands in addition to the major XO/XDH.
form. These bands can most likely be attributed to proteolytically cleaved fragments of the native enzyme, which are reported to possess oxidase but not dehydrogenase activity. It is interesting to note that despite an increase in protein expression after 12 hours, significant increases in XO activity and oxidase-dependent \( \text{O}_2^- \) generation were observed only after 30 hours. A possible explanation is that native XDH produces only very limited amounts of \( \text{O}_2^- \) and that conversion to the oxidase form is therefore necessary to facilitate \( \text{O}_2^- \) generation. This process is supported by ONOO\(^-\) and may therefore involve tyrosine nitration or oxidation of the enzyme. Indeed, protein tyrosine nitration was most pronounced 30 hours after LPS injection.

An NAD(P)H oxidase has been shown to be involved in vascular \( \text{O}_2^- \) generation under normal conditions as well as in pathophysiological states. Results of experiments using antisense cDNA directed against \( p22\text{phox} \) and depletion of the p67phox subunit of the oxidase by immunoabsorption suggested that an isoform of the leukocyte-type NAD(P)H oxidase expressed within the arterial wall is involved in the increased formation of \( \text{O}_2^- \) after treatment with angiotensin II. We observed expression of 3 components of the NAD(P)H oxidase, all of which were upregulated by LPS. The failure to detect \( p22\text{phox} \) and \( p47\text{phox} \) proteins but not \( p22\text{phox} \) mRNA expression in the rat aorta may be attributable to differences in the protein sequence of the subunits compared with human proteins. Indeed, when human cells were used, \( p22\text{phox} \) and \( p47\text{phox} \) were observed in Western blot analysis (R.P. Brandes, unpublished data, 1998).

Expression of \( gp91\text{phox} \) in nonphagocytic cells is controversial. The subunit has been detected in arteries and many cell types by Western blotting. With the use of reverse transcription-polymerase chain reaction, \( gp91\text{phox} \) was undetectable in fibroblasts but present in endothelial cells. In the present study, the anti-\( gp91\text{phox} \) antibody detected a nonglycosylated protein of \( \approx 75 \) kDa that was expressed at a low level under basal conditions but increased by LPS. Therefore, it appears possible that an isoform of \( gp91\text{phox} \) is expressed within the vessel wall, which shares a common epitope with the leukocyte-type NAD(P)H oxidase. Additional evidence of upregulation of NAD(P)H oxidase by LPS comes from tissue culture studies, in which tumor necrosis factor-\( \alpha \) and interleukin-1\( \beta \), which are released on LPS exposure, have been shown to increase NAD(P)H oxidase expression.

In addition to XO and NAD(P)H oxidase, \( iNOS \), which could be involved in \( \text{O}_2^- \) generation, was induced by LPS. It seems possible that \( iNOS \) contributes to \( \text{O}_2^- \) generation 12 hours after LPS treatment because expression of this enzyme peaked at this time point, whereas expression of XO and NADPH oxidase was maximal after 30 hours, when \( iNOS \) was nearly undetectable. This assumption is supported by the fact that \( NOR \) is sensitive to DPI. Nevertheless, up to now, \( iNOS \)-dependent \( \text{O}_2^- \) generation has been shown only for the \( \text{l}-\text{arginine} - \text{depleted enzyme} \), and it is unknown whether \( iNOS \) is able to produce \( \text{O}_2^- \) in vivo.

In addition to \( iNOS \) induction, LPS elicited a marked increase in MnSOD mRNA and protein expression. Despite the pronounced increase in \( \text{O}_2^- \) generation after LPS injection, the lucigenin signal was quite similar 12 and 30 hours after LPS injection, although a marked induction of XO and NADPH oxidase was apparent. The possible contribution of \( iNOS \)-dependent \( \text{O}_2^- \) formation could at least partially contribute to the \( \text{O}_2^- \) levels observed after 12 hours. Furthermore, the massive induction of MnSOD, which was most evident after 30 hours, facilitates a higher flux from \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) thus decreasing the level of \( \text{O}_2^- \) reacting with lucigenin.

Many factors may influence the time course of enzyme expression. Induction of \( iNOS \) mRNA after LPS treatment is rapid but transient, and turnover of the protein seems to be high. \( iNOS \) mRNA was detected 4 hours after LPS treatment but not after 16 hours (R.P. Brandes, unpublished data, 1998). In contrast, the increase in \( p22\text{phox} \) mRNA expression was much slower, not only after LPS treatment but also after in vivo exposure to angiotensin II. Although transcriptional control of \( iNOS \) has been extensively studied, for \( p22\text{phox} \) and XO, neither the promoter containing the transcription factor binding sites nor 5’-untranslated regions, message stability, and protein turnover time have been characterized. For \( iNOS \) and MnSOD, it has been shown that the redox-sensitive transcription factor NF-kB is involved in control of expression, at least in initiating gene transcription in response to oxidative stress. The fact that expression was not maintained during oxidative stress, however, may be related to many factors, including expression of inhibitory proteins or transient expression of 1 or more transcription factors required to induce full promoter activity.

An increase in both \( \text{O}_2^- \) and NO generation leads to formation of \( \text{ONOO}^- \). This strong oxidant, which nitrates protein tyrosine residues, has been implicated in vascular disturbances in endotoxemia. Indeed, we detected a pronounced increase in \( \text{ONOO}^- \) generation and protein tyrosine
To address the functional relevance of the observed increase in $O_2^-$ and ONOO$^-$ generation, vascular reactivity studies were performed. LPS treatment significantly impaired relaxation to SNP and acetylcholine via an effect that was not mediated by high steady-state levels of NO or $O_2^-$. Indeed, AMT, an inhibitor of iNOS, impaired relaxation to a similar extent in the LPS and control groups. This indicates that AMT itself partially impairs relaxation, potentially by inhibiting endothelial NOS, but also that iNOS-dependent NO formation does not directly contribute to endothelial dysfunction 30 hours after LPS injection.

Although increased generation of $O_2^-$ after LPS treatment was observed, $O_2^-$ did not seem to directly impair relaxation in the LPS model, because scavenging of $O_2^-$ did not restore but further impaired relaxation. Considering the data obtained, an explanation of how the $O_2^-$ scavengers used selectively impaired relaxation in LPS group can be only speculative. One reason might be potential side effects of the substances used. MnTBAP, eg, also scavenges peroxynitrite, which might be involved in endothelium-dependent relaxation. Furthermore, in addition to its $O_2^-$-scavenging properties, tiron also scavenges metal ions. $H_2O_2$ may play an important role in the effects of antioxidants. Dismutation of $O_2^-$ to the vasodilator $H_2O_2$ could contribute to the relaxation observed on addition of rhSOD. However, the metal ion–catalyzed decay of $H_2O_2$ in the Fenton reaction yields highly toxic hydroxyl radicals, which are involved in tissue toxicity of radicals and might therefore impair relaxation.

Whether ONOO$^-$, the reaction product of $O_2^-$ and NO, contributes to the impaired relaxation observed in the present study is difficult to determine. Because the inhibitors used also prevent ONOO$^-$ formation, an acute effect of this substance is rather unlikely. However, anti-nitrotyrosine Western analysis clearly demonstrates the accumulation of protein nitration over time. This nitration of protein tyrosine residues by ONOO$^-$ has been shown to inhibit different enzymes involved in endothelium-dependent relaxation, such as prostacyclin synthetase and NOS. In vivo, the half-life of nitrotyrosine bonds appears to be $>3$ days, indicating that constant low-level generation of ONOO$^-$ will lead to accumulation of tyrosine-nitrated protein. Considering the present observation, it is tempting to speculate that this accumulation of modified proteins is involved in the development of endothelial dysfunction after LPS treatment. Other toxic effects of peroxynitrite, such as hydroxyl radical formation, might also be important. Nevertheless, because of the lack of specific inhibitors and the abundance of naturally occurring inhibitors in vivo, such as uric acid, cysteine, glutathione, and vitamins, a causal involvement of ONOO$^-$ cannot be demonstrated and other mechanisms, such as activation of poly(ADP ribose) synthetase or disturbance of energy metabolism, have been shown to contribute to the development of endothelial dysfunction after LPS treatment. However, a role of ONOO$^-$ for these effects has also been suggested. Because of the lack of inhibitors, it is difficult to differentiate in vivo between a direct “toxic” effect of ONOO$^-$, a secondary effect of this substance due to hydroxyl radical formation, and hydroxyl radical formation from $H_2O_2$ via the Fenton reaction. Marked scavenging of hydroxyl radicals with substances such as mannitol or DMSO can be achieved only with very high concentrations of the scavenger. Nevertheless, in a recent study, diabetes-induced endothelial dysfunction of the rat aorta was improved by the hydroxyl radical scavenger dimethylthiourea. However, this substance also scavenges ONOO$^-$ (R.P. Brandes, unpublished data, 1998).

Using an experimental model of hypertension, we previously found that development of endothelial dysfunction required a concomitant increase of $O_2^-$ and NO production that resulted in a marked increase in tyrosine nitration. The increase in $O_2^-$ generation, which preceded the increase in NO and ONOO$^-$ formation, was not associated with the induction of endothelial dysfunction in the model used.

In another study, in which we determined the effect of aging on endothelium-dependent relaxation, we observed that aging was not only associated with impairment of acetylcholine-induced relaxation but also with impaired relaxation due to SOD. This SOD-induced relaxation was mediated by NO and was attributed to a decrease in the scavenging of basally generated NO by $O_2^-$. The fact that LPS impaired SOD-induced relaxation was surprising, because the amount of NO produced in rings from LPS-treated animals can be assumed to be much higher than that in rings from controls. It is therefore unlikely that $O_2^-$ directly contributes to the impairment of relaxation in the present study.

In summary, the impaired endothelium-dependent relaxation of the rat aorta after LPS treatment was associated with increased generation of $O_2^-$ by XO and NAD(P)H oxidase and an increase in ONOO$^-$ and nitrotyrosine formation. Because neither inhibition of $O_2^-$ nor inhibition of iNOS-dependent NO generation was sufficient to restore endothelium-dependent relaxation and because scavenging of $O_2^-$ further impaired relaxation in the LPS group, we propose that the prolonged formation of toxic products, such as $H_2O_2$ and ONOO$^-$, from $O_2^-$ and NO are potentially involved in the initiation of endothelial dysfunction after LPS treatment.

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