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 oxypurinol 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, oxypurinol, 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 aminohydromethylthiazine (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 Gruenenthal 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

Received December 21, 1998; first decision January 5, 1999; revision accepted January 11, 1999.
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Hypertension 1999;33:1243-1249

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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 F. Wientjes, University College London; and O.T.G. Jones, University of Bristol. 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 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. Luciferin (220 μmol/L) was used for O2 detection. 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 peroxynitrate-activated 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 μg/mL), pepstatin A (2 μg/mL), trypsin inhibitor (10 μg/mL), and leupeptin (2 μg/mL). After centrifugation at 29 000g for 20 minutes at 4°C, the supernatant containing the cytoplasmic fraction was removed and used for immunoblotting of p47phox, p67phox, 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. Western blotting was performed using their respective antibodies, which were linked positive controls for the NAD(P)H oxidase subunit proteins. Proteins were detected using the PC version of NIH-Image from Scion Corp.

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 butylmethylxO 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 luciferin. 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, acetylcholine (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 AMT12 (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 a band of ≈130 kDa along with the appearance of lower-molecular-weight bands of ≈70, 60, and 40 kDa, which was 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. Although a glycosylation smear of gp91phox was observed in a human leukocyte preparation, only a single band of ≈75 kDa was observed in rat aortic rings. p47phox and p22phox were undetectable in rat aorta using antibodies that recognized these proteins in human leukocytes. However, Northern blot...
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 LPS-treated rats.
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\textsubscript{2}^{-} formation for up to 30 hours by the induction XO and the “vascular-type” NAD(P)H oxidase. In addition, we observed a concomitant increase in MnSOD expression as well as ONOO\textsuperscript{-} and nitrotyrosine formation. LPS treatment impaired acetylcholine-induced relaxation. This effect was not mediated directly by \textit{O}, and rather than being beneficial, the scavenging of \textit{O}_{2}^{-} deteriorated relaxation.

A substantial portion of LPS-induced \textit{O}_{2}^{-} generation was sensitive to denudation of the rings. Nevertheless, whether this could be attributed to direct endothelial \textit{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 \textit{O}_{2}^{-} generation in the lucigenin assay. Moreover, the suitability of the lucigenin chemiluminescence method used here to determine \textit{O}_{2}^{-} generation, especially in endothelial cells, was recently questioned. Therefore, \textit{H}_{2}\textit{O}_{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 \textit{O}_{2}^{-} on exposure to LPS, the prolonged generation of \textit{O}_{2}^{-} requires induction of \textit{O}_{2}^{-}-generating enzymes. There are a number of possible enzymatic sources of \textit{O}_{2}^{-} in the vascular wall. The observation that the LPS-induced \textit{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

![Figure 4](image4.png)

**Figure 4.** Effect of LPS on MnSOD expression. Top, Northern blot analysis. ▼ indicates the different MnSOD transcripts obtained from alternative splicing. Bottom, Western blot analysis of aortic segments from control and LPS-treated rats. *P<0.05.

![Figure 5](image5.png)

**Figure 5.** Effect of LPS on iNOS expression, peroxynitrite (ONOO\textsuperscript{-}), and nitrotyrosine formation in rat aortic tissue. Top, iNOS expression as detected by Western blot analysis. Middle, Western blot showing the effect of LPS treatment on protein tyrosine nitration in aortic samples from control and LPS-treated rats 12 and 30 hours after injection. Bottom, Vascular peroxynitrite generation in control rings and rings removed 30 hours after LPS treatment. *P<0.05 (n=12).

![Figure 6](image6.png)

**Figure 6.** Effect of rhSOD and acetylcholine on preconstricted aortic rings. A, Relaxation induced by rhSOD (1 μmol/L) in preconstricted arteries from controls (●) and rats killed 12 (□) and 30 hours (■) after LPS treatment. B and C, Effect of rhSOD on acetylcholine-induced relaxation in rings from control (● and ○) and LPS-treated rats (■ and □) 12 (B) and 30 (C) hours after injection. Open and closed symbols represent responses obtained in the presence and absence, respectively, of the inhibitors used. *P<0.05 vs the respective untreated group (n=5).
The enzyme.21 Indeed, protein tyrosine nitration was most pronounced 30 hours after LPS injection.

In addition, 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 $O_2^-$ formation could at least partially contribute to the $O_2^-$ levels observed after 12 hours. Furthermore, the massive induction of MnSOD, which was most evident after 30 hours, facilitates a higher flux from $O_2^-$ to $H_2O_2$, thus decreasing the level of $O_2^-$ 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 $p22phox$ mRNA expression was much slower, not only after LPS treatment but also after in vivo exposure to angiotensin II.30 Although transcriptional control of iNOS has been extensively studied,31 for $p22phox$ 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 iNOS31 and MnSOD,32 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.33

An increase in both $O_2^-$ and NO generation leads to formation of ONOO$^-$,34,35 This strong oxidant, which nitrates protein tyrosine residues, has been implicated in vascular disturbances in endotoxemia.36,37 Indeed, we detected a pronounced increase in ONOO$^-$ generation and protein tyrosine...

**Figure 7.** Effect of AMT, tiron, MnTBAP, and oxypurinol on relaxant responses to acetylcholine in U44069-precontracted aortic rings from control rats (■ and □) and rats treated with LPS 30 hours after injection (◆ and △). Open and closed symbols represent responses obtained in the presence and absence, respectively, of the inhibitors used. *P<0.05 vs the respective untreated group (n=10).
nitrination after LPS injection. However, the pathophysiologically
consequences of ONOO\textsuperscript{−} formation and nitrotyrosine
formation are controversial.\textsuperscript{34,37}

To address the functional relevance of the observed in-
crease in O\textsubscript{2}\textsuperscript{−} and ONOO\textsuperscript{−} 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\textsubscript{2}\textsuperscript{−}. 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 inhib-
itating endothelial NOS, but also that iNOS-dependent NO
formation does not directly contribute to endothelial dysfunc-
tion 30 hours after LPS injection.

Although increased generation of O2\textsuperscript{−} after LPS treatment
was observed, O2\textsuperscript{−} did not seem to directly impair relaxation in
the LPS model, because scavenging of O2\textsuperscript{−} did not restore but
further impaired relaxation. Considering the data obtained, an
explanation of how the O2\textsuperscript{−} scavengers used selectively impaired
relaxation in LPS group can be only speculative. One reason
might be potential side effects of the substances used. MnTBAP,
e.g., also scavenges peroxynitrite,\textsuperscript{38} which might be involved in
endothelium-dependent relaxation.\textsuperscript{36,39} Furthermore, in addition
to its O2\textsuperscript{−}-scavenging properties, tiron also scavenges metal
ions.\textsuperscript{40} H2O\textsubscript{2} may play an important role in the effects of
antioxidants. Dismutation of O2\textsuperscript{−} to the vasodilator H2O\textsubscript{2} could
contribute to the relaxation observed on addition of rhSOD.
However, the metal ion–catalyzed decay of H2O\textsubscript{2} in the Fenton
reaction yields highly toxic hydroxyl radicals, which are
involves in tissue toxicity of radicals\textsuperscript{41} and might therefore impair
relaxation.

Whether ONOO\textsuperscript{−}, the reaction product of O2\textsuperscript{−} and NO,
contributes to the impaired relaxation observed in the present
study is difficult to determine. Because the inhibitors used also
prevent ONOO\textsuperscript{−} 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\textsuperscript{−} has
been shown to inhibit different enzymes involved in endothel-
ium-dependent relaxation, such as prostacyclin synthetase\textsuperscript{42} and
NOS.\textsuperscript{43} In vivo, the half-life of nitrotyrosine bonds appears to be
>3 days,\textsuperscript{44} indicating that constant low-level generation of
ONOO\textsuperscript{−} 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,\textsuperscript{45} 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, gluta-
thione, and vitamins,\textsuperscript{37} a causal involvement of ONOO\textsuperscript{−} cannot
be demonstrated and other mechanisms, such as activation of
poly(ADP ribose) synthetase\textsuperscript{46} or disturbance of energy metab-
olism,\textsuperscript{47} have been shown to contribute to the development of
endothelial dysfunction after LPS treatment. However, a role of
ONOO\textsuperscript{−} for these effects has also been suggested.\textsuperscript{37,47} Because
of the lack of inhibitors, it is difficult to differentiate in vivo
between a direct “toxic” effect of ONOO\textsuperscript{−}, a secondary effect of
this substance due to hydroxy radical formation, and hydroxyl
radical formation from H2O\textsubscript{2} via the Fenton reaction. Marked
scavenging of hydroxyl radicals with substances such as man-
nitol or DMSO can be achieved only with very high concentra-
tions of the scavenger. Nevertheless, in a recent study,\textsuperscript{48}
diabetes-induced endothelial dysfunction of the rat aorta was
improved by the hydroxyl radical scavenger dimethylthiourea.
However, this substance also scavenges ONOO\textsuperscript{−} (R.P. Brandes,

Using an experimental model of hypertension, we previ-
ously found that development of endothelial dysfunction
required a concomitant increase of O2\textsuperscript{−} and NO production that
resulted in a marked increase in tyrosine nitration. The increase in O2\textsuperscript{−} generation, which preceded the increase in
NO and ONOO\textsuperscript{−} formation, was not associated with the
induction of endothelial dysfunction in the model used.\textsuperscript{39}

In another study, in which we determined the effect of
aging on endothelium-dependent relaxation,\textsuperscript{50} we observed
that aging was not only associated with impairment of
acetylcholine-induced relaxation but also with impaired re-
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 O2\textsuperscript{−}. The fact that
LPS impaired SOD-induced relaxation was surprising, be-
cause 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 O2\textsuperscript{−} directly
contributes to the impairment of relaxation in the present
study.

In summary, the impaired endothelium-dependent relax-
lation of the rat aorta after LPS treatment was associated with
increased generation of O2\textsuperscript{−} by XO and NAD(P)H oxidase
and an increase in ONOO\textsuperscript{−} and nitrotyrosine formation.
Because neither inhibition of O2\textsuperscript{−} nor inhibition of iNOS-
dependent NO generation was sufficient to restore endothe-
lum-dependent relaxation and because scavenging of O2\textsuperscript{−}
further impaired relaxation in the LPS group, we propose that
the prolonged formation of toxic products, such as H2O\textsubscript{2} and
ONOO\textsuperscript{−}, from O2\textsuperscript{−} and NO are potentially involved in the
initiation of endothelial dysfunction after LPS treatment.

Acknowledgments

Dr Brandes received a research grant from the ADUMED Founda-
tion and a Young Investigator grant from Klinikum der Johann
Wolfgang Goethe-Universität. We thank Dr Ingrid Fleming for
helpful suggestions in preparing the manuscript.

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Hypertension. 1999;33:1243-1249
doi: 10.1161/01.HYP.33.5.1243

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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