ARTHRITIS & RHEUMATISM
Vol. 54, No. 6, June 2006, pp 1847–1855
DOI 10.1002/art.21891

Endothelial Dysfunction in Rat Adjuvant-Induced Arthritis

Vascular Superoxide Production by NAD(P)H Oxidase and Uncoupled Endothelial Nitric Oxide Synthase

Yoshisuke Haruna,[1] Yoshitaka Morita,[1] Norio Komai,[1] Toyotaka Yada,[1] Takeo Sakuta,[1] Naruya Tomita,[1] David A. Fox,[2] and Naoki Kashihara[1]

Objective. **To investigate endothelial function and levels of vascular oxidative stress in rat adjuvant-induced arthritis (AIA), in view of mounting evidence for an association between rheumatoid arthritis (RA) and accelerated vascular disease.**

Methods. **Thoracic aortic rings were prepared from AIA and control rats. After preconstriction by norepinephrine, the vasodilatory response to acetylcholine was determined. The amounts of 4-hydroxy-2-nonenal (HNE) and nitrotyrosine in AIA rat aortas were measured by Western blotting. Homogenates of the aortas were incubated with various substrates for superoxide-producing enzymes, and superoxide production was assessed by fluorogenic oxidation of dihydroethidium to ethidium. Expression of endothelial nitric oxide synthase (eNOS) in aortas was examined by real-time reverse transcriptase–polymerase chain reaction and Western blotting. Serum levels of tetrahydrobiopterin (BH_4), a critical eNOS cofactor, were determined by high-performance liquid chromatography.**

Results. **Endothelium-dependent relaxation of the aortic ring was significantly depressed in AIA rats compared with control rats. The amounts of HNE and nitrotyrosine were increased in AIA rat aortas, indicating overproduction of reactive oxygen species. Incubation of AIA rat aorta homogenates with NADH or L-arginine, a substrate of eNOS, resulted in a significant increase in superoxide production. Endothelial NOS was highly expressed in AIA rat aortas. Serum levels of BH_4 were significantly lower in AIA. Treatment of AIA with BH_4 reversed the endothelial dysfunction, suggesting that its deficiency may contribute to the uncoupling of eNOS.**

Conclusion. **Vascular dysfunction in RA can be partially modeled in animals. NAD(P)H oxidase and uncoupled eNOS are responsible for the increase in vascular oxidative stress, which is likely to be involved in the endothelial dysfunction in AIA.**

Rheumatoid arthritis (RA) is a common chronic disease characterized by persistent inflammation of multiple joints. RA is associated with an increased mortality from cardiovascular causes, which exceeds that of the general population (1–3). Recent reports demonstrate that endothelial function is reduced in RA patients with high inflammatory activity (4–6). Although the underlying mechanisms for endothelial dysfunction in RA are poorly understood, it is postulated that systemic inflammation may be involved in the early vascular damage (7–9). In support of this hypothesis, anti–tumor necrosis factor (anti-TNF) therapy has been shown to improve endothelial dysfunction in patients with active RA (6,10).

Endothelium plays a pivotal role in the regulation of vascular tone and structure through the release of various vasoactive agents, such as vasodilators and vasoconstrictors. It has been recognized that alterations of endothelial function are involved in the development and progression of atherosclerosis and its clinical com-

Dr. Morita is recipient of the 2005 Research Award from the Kawasaki Medical Foundation.

[1]Yoshisuke Haruna, MD, Yoshitaka Morita, MD, Norio Komai, MD, Toyotaka Yada, MD, Takeo Sakuta, MD, Naruya Tomita, MD, Naoki Kashihara, MD: Kawasaki Medical School, Kurashiki, Japan; [2]David A. Fox, MD: University of Michigan, Ann Arbor.

Address correspondence and reprint requests to Yoshitaka Morita, MD, Division of Nephrology and Rheumatology, Department of Internal Medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan. E-mail: morita@med.kawasaki-m.ac.jp.

Submitted for publication July 5, 2005; accepted in revised form February 21, 2006.

plications (11,12). Endothelial dysfunction is considered to represent reduced bioavailability of nitric oxide (NO), which is a major endothelium-dependent vasodilator. Endothelium-dependent NO is also known to have other antiatherosclerotic properties, including inhibition of cell growth, leukocyte adhesion, and platelet adherence and aggregation (11). Regulatory mechanisms that control vascular NO bioavailability in pathophysiologic states are complex. It has been reported that vascular production of reactive oxygen species (ROS), such as superoxide (O_2^-), is increased in hypertension, atherosclerosis, or diabetes (13,14). O_2^- reacts rapidly with NO, resulting in the formation of peroxynitrite ($ONOO^-$), which could lead to a loss of bioactivity of NO. The increased oxidative stress may affect the synthesis of NO. Vascular NO is mainly synthesized by endothelial NO synthase (eNOS) from the precursor L-arginine. However, previous data demonstrate that increased oxidative stress promotes a dysfunctional eNOS, which generates O_2^- instead of NO (15). This dysfunctional enzyme is termed uncoupled eNOS. It is reported that the formation of uncoupled eNOS is linked to oxidative degradation of the critical eNOS cofactor tetrahydrobiopterin (BH_4) (16,17).

Animal models have been essential to understanding the pathophysiologic mechanisms of human diseases. To elucidate the mechanism of endothelial dysfunction caused by systemic inflammation, we used rat adjuvant-induced arthritis (AIA) in the rat, which has been widely used as a model of RA. The experiments conducted in the study were designed to 1) determine whether endothelium-dependent relaxation is impaired in the vasculature of the arthritis model, as shown in patients with RA; 2) determine whether vascular oxidative stress is increased in AIA; and 3) identify the source of vascular ROS production if it is increased.

MATERIALS AND METHODS

Induction of rat AIA. Six-week-old male Lewis rats were obtained from Charles River Japan (Kanagawa, Japan). Complete adjuvant was prepared by suspending heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) in mineral oil at 10 mg/ml. Rats were injected intradermally with 100 μl of the adjuvant at the base of the tail. The animals developed arthritis by day 10 post–adjuvant injection. Twenty-one days after the onset of arthritis, rats were killed and the thoracic aortas were isolated for further experiments. In a separate experiment, BH_4 (20 mg/kg; Sigma-Aldrich Japan, Tokyo, Japan) was injected intraperitoneally into AIA rats from day 14 to day 20 after the onset of arthritis. All procedures were performed in accordance with our institutional guidelines for animal research.

Endothelium-dependent vascular responses in rat AIA. Thoracic aortic ring preparations were obtained from AIA and control rats. Cylindrical 3.0-mm–long segments were cut from the aorta and were bathed in 5 ml of Krebs bicarbonate saline (120 m*M* NaCl, 5.2 m*M* KCl, 2.4 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 25 m*M* $NaHCO_3$, 0.03 m*M* Na_2-EDTA, and 11 m*M* dextrose [pH 7.4]) equilibrated with 95% O_2 and 5% CO_2, and maintained at 37°C. The rings were suspended under 1*g* of tension and preconstricted by adding $3 \times 10^{-7}M$ norepinephrine. After the contraction force had reached a plateau, acetylcholine ($10^{-9}M$ to $10^{-5}M$; endothelium-dependent vasodilator) or sodium nitroprusside ($10^{-9}M$ to $10^{-5}M$; endothelium-independent vasodilator) was added incrementally to the bath. The force of isometric contraction was measured using a force-displacement transducer (Model MTOB-1Z; Labo Support, Osaka, Japan). Responses to acetylcholine were expressed as the percentage of the precontracted tension induced by norepinephrine.

Western immunoblotting. Isolated aorta samples were homogenized in lysis buffer containing 250 m*M* sucrose, 50 m*M* dithiothreitol, 3 m*M* HEPES (pH 7.9), 0.5 m*M* EGTA, 1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, 14 μM (4-guanidino)butane, and 1% Tween 20. Homogenates were centrifuged at 8,000*g* for 10 minutes at 4°C to remove tissue debris. The supernatant was used for detection of 4-hydroxy-2-nonenal (HNE)–modified proteins, nitrotyrosine, and different isotypes of NOS in the same manner as described previously (18,19). Briefly, for the HNE immunoblot, proteins (50 μg) were separated in a 7.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated for 1 hour with anti-HNE monoclonal antibody (7 μg/ml; Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan) or antiactin monoclonal antibody (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation for 1 hour with peroxidase-conjugated goat anti-mouse IgG antibody (0.1 μg/ml).

In control samples, anti-HNE antibody was preincubated for 2 hours with HNE-modified bovine serum albumin (BSA), which was prepared by incubating 10 mg/ml BSA with HNE (1 m*M*; OXIS International, Portland, OR) at 37°C for 2 hours. Bands were visualized using the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences, Tokyo, Japan) and normalized to the quantity of actin protein using image analysis software (NIH Image, National Institutes of Health, Bethesda, MD; online at: http://rsb.info.nih.gov/nih-image/).

For immunoblot analysis of nitrotyrosine or NOS isotype proteins, 100-μg protein samples were separated using 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. The sample-transferred membranes were incubated for 2 hours with rabbit polyclonal antibody against nitrotyrosine (1 μg/ml; Upstate Biotechnology, Lake Placid, NY) or various isotypes of NOS, including eNOS, neuronal NOS (nNOS), and inducible NOS (iNOS) (0.1 μg/ml each; Santa Cruz Biotechnology). As a specificity control, antinitrotyrosine antibody was preincubated with nitrotyrosine (10 m*M*; Cayman Chemical, Ann Arbor, MI). The membranes were then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (0.08 μg/ml). For immunoblot analysis of

nitrotyrosine or monomers of NOS, samples were heated at 95°C for 3 minutes before electrophoresis. For immunoblot analysis of the dimeric form of eNOS, samples were not heated and the temperature of the gel was maintained below 15°C during electrophoresis (low-temperature SDS-PAGE) as described elsewhere (18).

Fluorescence spectrometric assay of O_2^- production from isolated aortas. The isolated aortas were homogenized with a glass homogenizer in ice-cold buffer containing 25 m*M* HEPES, 1 m*M* EDTA, and 0.1 m*M* phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 6,000*g* for 5 minutes at 4°C, the supernatant containing the membrane and cytosolic components was separated. Fluorescence spectrometry of O_2^- production from the homogenates was performed as described previously (18). The fluorogenic oxidation of dihydroethidium to ethidium was used as a measure of O_2^-. The homogenates (20 μg) were incubated at 37°C for 30 minutes with dihydroethidium (0.02 m*M*; Sigma-Aldrich Japan), salmon testes DNA (0.5 mg/ml), and various substrates for superoxide-producing enzymes and their inhibitors in a microtiter plate placed away from direct light. Ethidium-DNA fluorescence was measured at an excitation of 480 nm and an emission of 610 nm by using a fluorescence microplate reader (Fluostar Optima; Moritex Bio-Science, Tokyo, Japan). A blank without the homogenate was used to measure background fluorescence, and its level was subtracted from each sample. The enzyme activities of different pathways are expressed relative to the control.

The following substrates or inhibitors were used in this study. NADH (0.1 m*M*) was used as a substrate for NAD(P)H oxidases (enzymes that use NADH or NADPH as a substrate). Diphenylene iodonium chloride (0.1 m*M*) was added to inhibit NAD(P)H oxidases. L-arginine (1 m*M*) was used as a substrate for NOS. N^G-nitro-L-arginine methyl ester (L-NAME; 1 m*M*) was used to block NOS activity. The effect of BH_4 (0.01 m*M*) on L-arginine–induced O_2^- production was also examined. Xanthine (0.1 m*M*) was used as a substrate for xanthine oxidase. Succinate (5 m*M*) was used as a substrate for intramitochondrial O_2^- production, and antimycin (0.05 m*M*) was used to block the normal reaction in the respiratory chain.

RNA isolation and real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Total cellular RNA was extracted with TRIzol in a single-step method. RT reactions were performed using a Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences) for the first-strand complementary DNA (cDNA) synthesis. Real-time quantitative PCR was performed using an ABI Prism 7700 sequence-detection system (Applied Biosystems, Foster City, CA). The sequences of primers and probes specific for eNOS, nNOS, and iNOS have been described previously (18). Complementary DNA from serially diluted samples was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems). The threshold cycle is the PCR cycle at which an increase in the fluorescent emission above the baseline signal is first detected. Since the values of the threshold cycle decrease proportionally with increased target quantity, they were used to determine the relative cDNA amounts in each sample and to evaluate levels of eNOS, nNOS, and iNOS messenger RNA (mRNA), which were normalized to the quantity of GAPDH mRNA, as described previously (18,20).

Determination of serum BH_4 concentrations. Serum concentrations of BH_4 were determined by high-performance liquid chromatography (HPLC) as described elsewhere (21,22). Serum samples were collected 21 days after the onset of AIA and mixed at a ratio of 1:1 with a solution of 0.5*M* perchloric acid containing 0.1 m*M* Na_2-EDTA and 0.1 m*M* $Na_2S_2O_3$ for protein separation. After filtration, BH_4 concentrations in the samples were measured by HPLC. BH_4 was detected fluorometrically at wavelengths of 350 nm for excitation and 440 nm for emission by postcolumn $NaNO_2$ oxidation with a reversed-phase ion-pair LC system (LC-10 series; Shimadzu, Kyoto, Japan).

Immunohistochemistry. Frozen tissue samples of the aorta were cut into 4-μm sections and incubated in methanol with 3% H_2O_2 to block endogenous peroxidase activity. Sections were incubated with mouse anti–rat endothelial cell antigen 1 monoclonal antibody (Cosmo Bio, Tokyo, Japan). For negative controls, a monoclonal mouse IgG1 (Santa Cruz Biotechnology) was used at equivalent concentrations. The primary antibody was detected using a Histofine Simple Stain Max PO (Multi) kit and 3,3′-diaminobenzidine (Sigma-Aldrich Japan).

Statistical analysis. Data are expressed as the mean ± SEM of the indicated number of samples studied. The Wilcoxon signed rank test was used to analyze matched pairs. The Mann-Whitney U test was used to compare group means.

RESULTS

Endothelium-dependent relaxation of aortic rings in response to acetylcholine is depressed in rat AIA. To determine whether endothelial function is disturbed in AIA, thoracic aortas were isolated 21 days after the onset of arthritis. At that point, clinical arthritis had reached its apparent maximum in most of the animals, and the mean body weight was 94% of that of control rats. The isolated aortas were cut into 3.0-mm long rings and placed in organ chambers. Norepinephrine was applied to achieve near-maximal contraction, and dose responses to various concentrations of acetylcholine were determined.

We found that the endothelium-dependent relaxation of the aortic ring was significantly depressed ($P < 0.01$) in AIA rats compared with control rats (Figure 1). The mean vasodilation in response to $10^{-7}M$, $10^{-6}M$, and $10^{-5}M$ of acetylcholine, which was administered subsequent to the norepinephrine-induced preconstriction, was 13.1%, 34.4%, and 56.0%, respectively, in AIA rats and 55.7%, 85.7%, and 99.6%, respectively, in control rats. To determine whether endothelial cells were histologically damaged, expression of rat endothelial cell antigen 1 in the isolated aortas was examined. The endothelial cells were found to be preserved in AIA (results not shown). Tissue

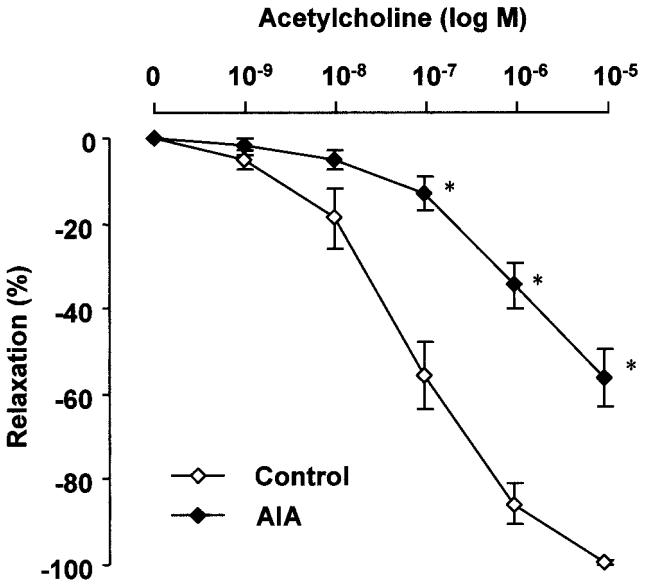

Figure 1. Depression of endothelial function in rat adjuvant-induced arthritis (AIA). Thoracic aortas were isolated from rats with AIA 21 days after the onset of disease. The aortic rings obtained from AIA and control rats were placed in organ chambers. Norepinephrine was applied to achieve near-maximal contraction, and dose responses to the indicated concentrations of acetylcholine were determined. Relaxation was expressed as the percentage of the precontracted tension induced by norepinephrine. Values are the mean ± SEM (n = 6 rats per group). ∗ = $P < 0.01$ versus control.

sections were also stained with hematoxylin and eosin, but no histologic changes were observed (results not shown).

Increased oxidative stress in the aortas of AIA rats. We subsequently studied the levels of ROS production in the isolated aortas. First, the HNE content, an index of lipid peroxidation, was examined by Western blotting. Results revealed that the amounts of HNE-modified proteins were significantly (2.84-fold) higher ($P < 0.05$) in the aortas of AIA rats than in control rat aortas (Figure 2A). We next examined vascular formation of nitrotyrosine, which is considered to be an indicator of $ONOO^-$, a strong oxidant. Western blot analysis revealed that nitrotyrosine-containing proteins were significantly (1.72-fold) ($P < 0.05$) increased in the aortas of AIA rats compared with those of control rats (Figure 2B). The specific reactivity of each antibody on Western blots was confirmed by the data showing that preincubation of each antibody with HNE-modified albumin or nitrotyrosine blocked its reactivity (Figure 2).

NAD(P)H oxidase and NOS are responsible for O_2^- production in the aortas of AIA rats. To investigate the source of vascular ROS production, homogenates of the isolated aortas were incubated with or without a variety of substrates, and O_2^- production was measured by a fluorescence spectrometric assay (Figure 3). Con-

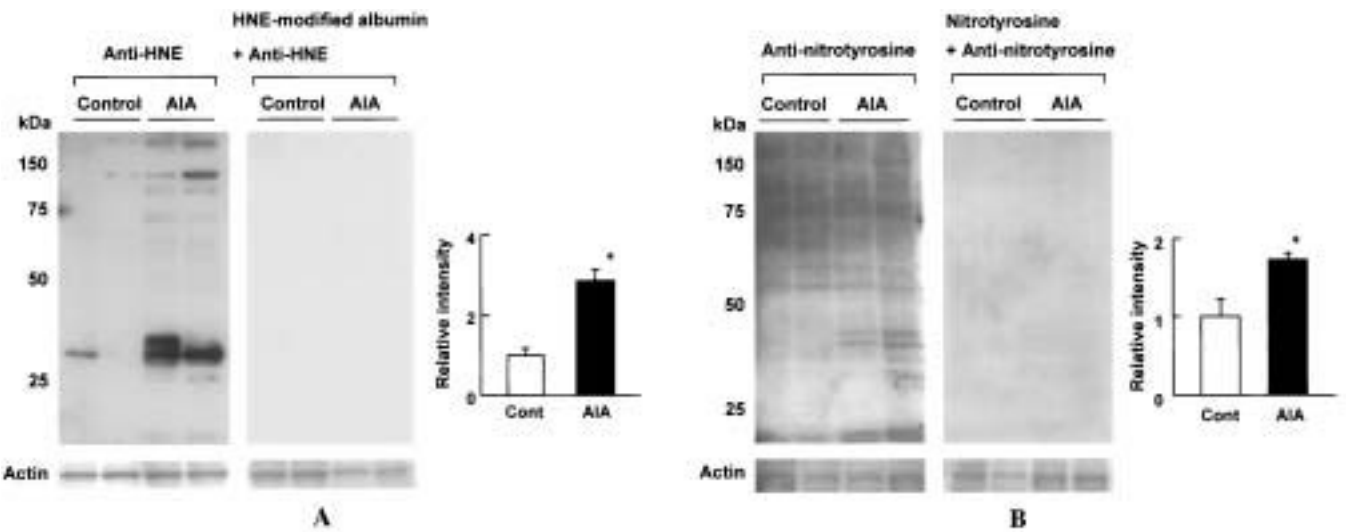

Figure 2. Increased oxidative stress in aortas of rats with adjuvant-induced arthritis (AIA). Shown are the results of Western blot analyses of oxidatively modified proteins containing 4-hydroxy-2-nonenal (HNE) (**A**) and nitrotyrosine (**B**) in isolated aortas from control and AIA rats. In control samples, anti-HNE antibody or antinitrotyrosine was preincubated with HNE-modified bovine serum albumin or nitrotyrosine, respectively, before incubation with the membranes. Representative blots are shown. The bands were normalized to the quantity of actin protein using National Institutes of Health image analysis software. Values are the mean and SEM (n = 5 rats per group for HNE; n = 6 rats per group for nitrotyrosine) and are expressed relative to the control. ∗ = $P < 0.05$ versus control.

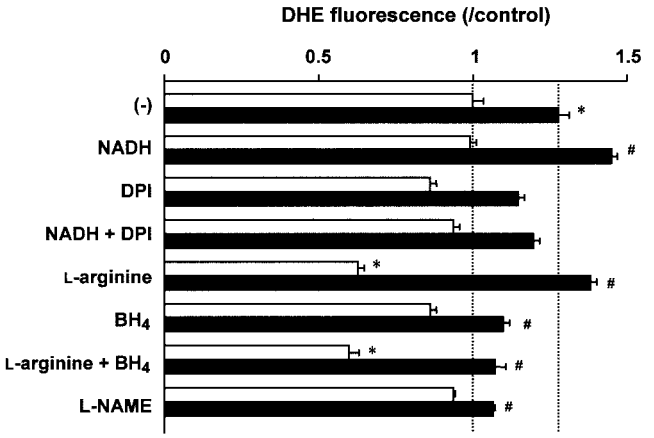

Figure 3. NAD(P)H oxidase and nitric oxide synthase (NOS) are responsible for superoxide (O_2^-) production in the aortas of rats with adjuvant-induced arthritis (AIA). Homogenates of isolated aortas from control rats (open bars; n = 4 rats per group) and AIA rats (solid bars; n = 5) were incubated with NADH or L-arginine with or without inhibitors of the corresponding enzymes, and O_2^- production was measured by fluorogenic oxidation of dihydroethidium (DHE) to ethidium. Diphenylene iodonium chloride (DPI) was added to inhibit NAD(P)H oxidase. N^G-nitro-L-arginine methyl ester (L-NAME) was used to block NOS activity. The effect of tetrahydrobiopterin (BH_4) on L-arginine–induced O_2^- production was also examined. Values are the mean and SEM and are expressed relative to the control. * = $P < 0.05$ versus untreated control rat aortas; # = $P < 0.05$ versus AIA rat aortas without substrates. (–) = untreated control rat aortas or AIA rat aortas without substrates.

sistent with the data shown in Figure 2, spontaneous production of O_2^- was significantly higher in AIA rats than in control rats ($P < 0.05$). Incubation of homogenates of AIA rat aortas with NADH resulted in a significant increase in O_2^- production compared with incubation without a substrate ($P < 0.05$). The enhanced activity of NAD(P)H oxidase in AIA rat aortas was confirmed by the finding that diphenylene iodonium chloride inhibited NADH-induced O_2^- overproduction. Importantly, L-arginine, a substrate of NOS, increased O_2^- formation by AIA rat aortas ($P < 0.05$), but L-arginine decreased O_2^- formation in control rat aortas ($P < 0.05$ versus untreated control rat aortas). Consistent with this finding, L-NAME, a NOS inhibitor, decreased O_2^- production by AIA rat aortas ($P < 0.05$). In contrast, substrates for xanthine oxidases and mitochondrial respiratory chain enzymes did not increase the O_2^- production (results not shown). Taken together, these results suggest that NAD(P)H oxidase and NOS are the predominant sources of O_2^- production in the aortas of AIA rats.

Expression of NOS in the aortas of AIA rats. We investigated levels of NOS mRNA expression in isolated aortas by real-time RT-PCR (Figure 4). Endothelial NOS mRNA expression levels were significantly higher in AIA rat aortas than in control rat aortas (2.75-fold) ($P < 0.05$). In contrast, no difference was observed in nNOS and iNOS mRNA expression. We further examined the levels of NOS protein in the isolated aortas by Western blotting. Consistent with the results of mRNA analysis, the immunoreactivity of total eNOS protein was significantly higher in AIA rat aortas (1.81-fold) ($P < 0.05$), whereas there was no significant difference in levels of nNOS and iNOS protein (Figure 5A). Endothelial NOS protein is composed of 2 identical subunits. Previous studies have demonstrated 2 bands for eNOS protein, representing a 130-kd eNOS monomer and a 260-kd eNOS dimer, when low-temperature SDS-PAGE is performed under reducing conditions (15,18). The immunoblot analysis after low-temperature SDS-PAGE revealed the expected 2 bands for eNOS protein in the isolated aortas. Of note, the dimer: monomer expression ratio was significantly lower ($P < 0.05$) in AIA rat aortas than in control rat aortas (Figure 5B).

Association of BH_4 deficiency with endothelial dysfunction in AIA. BH_4 is an essential cofactor of eNOS, and its deficiency is reported to decrease the bioavailability of NO. Serum levels of BH_4 were measured by HPLC. Mean ± SEM serum BH_4 levels in AIA rats (11.1 ± 1.3 ng/ml, n = 8) were significantly lower than those in control rats (19.9 ± 4.5 ng/ml, n = 7) ($P < 0.05$). Moreover, as shown in Figure 3, coincubation of homogenates of the AIA rat aortas with BH_4 reversed

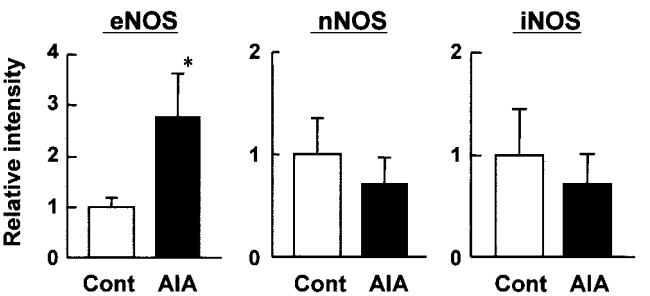

Figure 4. Expression of nitric oxide synthase (NOS) mRNA in isolated aortas from rats with or without adjuvant-induced arthritis (AIA). Real-time reverse transcriptase–polymerase chain reaction was performed as described in Materials and Methods. Levels of endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) mRNA were normalized to the GAPDH mRNA level. Values are the mean and SEM (n = 5 rats per group) and are expressed relative to the control. * = $P < 0.05$ versus control.

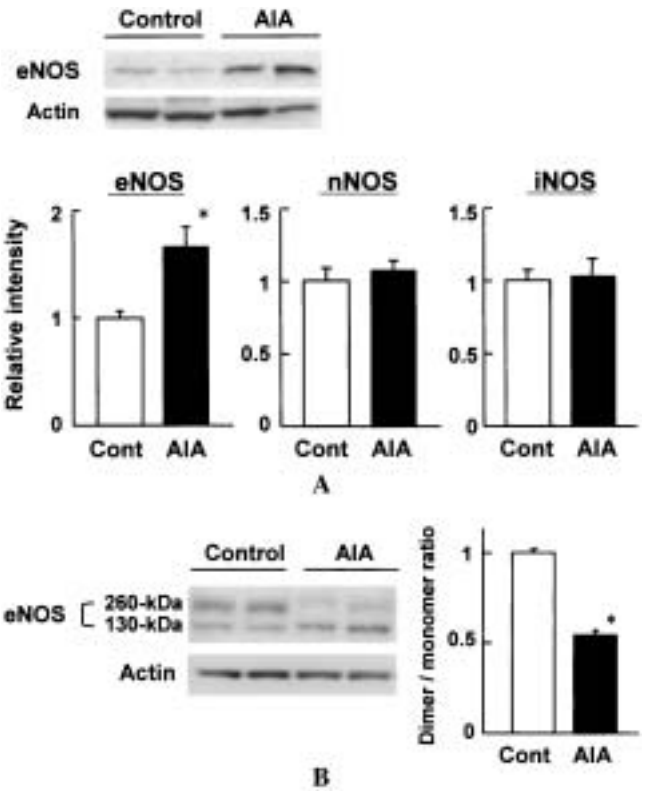

Figure 5. Western blot analysis of NOS in isolated aortas. **A,** Conventional immunoblotting for eNOS, nNOS, and iNOS in isolated aortas from AIA and control rats. Representative blots for eNOS are shown. Densitometric quantification of the corresponding bands was performed using National Institutes of Health (NIH) image analysis software. Values are the mean and SEM (n = 6 rats per group) and are expressed relative to the control. **B,** Representative blots for eNOS after low-temperature sodium dodecyl sulfate–polyacrylamide gel for electrophoresis. The intensity of dimers and monomers was determined with NIH image analysis software, and the results are expressed as the dimer:monomer ratio. Values are the mean and SEM (n = 4 rats per group) and are expressed relative to the control. $* = P < 0.05$ versus control. See Figure 4 for other definitions.

L-arginine–induced O_2^- overproduction. We further explored whether administration of BH_4 improves the endothelial dysfunction in AIA. AIA rats were given BH_4 by intraperitoneal injection daily for 7 days before they were killed, and endothelium-dependent and -independent vascular responses were determined. We found that treatment with BH_4 reversed the endothelial dysfunction in AIA ($P < 0.01$) (Figure 6A). The severity of arthritis was not affected by this treatment.

DISCUSSION

Endothelial dysfunction is characterized by reduced vasodilation, a proinflammatory state, and prothrombotic properties (11). It is recognized to represent the earliest stage of atherosclerosis. Recently, endothelial dysfunction has been reported in patients with RA with high levels of inflammatory activity (4–6), suggesting that systemic inflammation may contribute to the vascular dysfunction, resulting in increased mortality from cardiovascular causes. Although interest has focused on the issue of atherosclerosis in RA, there is little information regarding vascular function in experimental models of arthritis. We thus investigated endothelial function in rat AIA. The data revealed that endothelial function in arthritic rats is significantly depressed without any histologic damage, suggesting that the endothelium-derived NO release is impaired before the development of overt atherosclerosis. Rat AIA is characterized by an acute self-limited disease. Nevertheless, our results indicate that vascular dysfunction in RA can be partially modeled in animals and occurs early in disease.

The pathophysiology of endothelial dysfunction is complicated and appears to involve multiple mechanisms (11). Accumulating evidence suggests that increased vascular production of ROS plays an important role (14). We hypothesized that vascular ROS production is increased in AIA, and we obtained several pieces of evidence to support this hypothesis. First, the amount of HNE-modified proteins was higher in isolated aortas from AIA rats than in those from control rats. Second, vascular production of nitrotyrosine, which is the footprint of NO interaction with ROS, was greater in AIA. Third, O_2^- production from isolated aortas, as detected by conversion of dihydroethidium to ethidium, was increased in AIA rats compared with control rats. On the basis of these results, we conclude that vascular oxidative stress is increased in the AIA model. In the vascular wall, ROS can be generated in the endothelium as well as by smooth muscle cells and fibroblasts (23,24). It should be noted that the present study did not identify cellular sources of ROS. Vascular ROS are generated by virtually all types of vascular cells and potentially affect every cell type in the vascular wall (23,24). Thus, it is plausible that increased vascular oxidative stress contributes to endothelial dysfunction in AIA.

Several production pathways could account for the vascular ROS increase in AIA. We found that incubation of homogenates of AIA aortas with NADH results in a significant increase in O_2^- production, suggesting that NAD(P)H oxidase is one of the important sources of the excessive ROS production. NAD(P)H oxidases have been proposed to be major sources of oxidative excess in the vasculature (23,25).

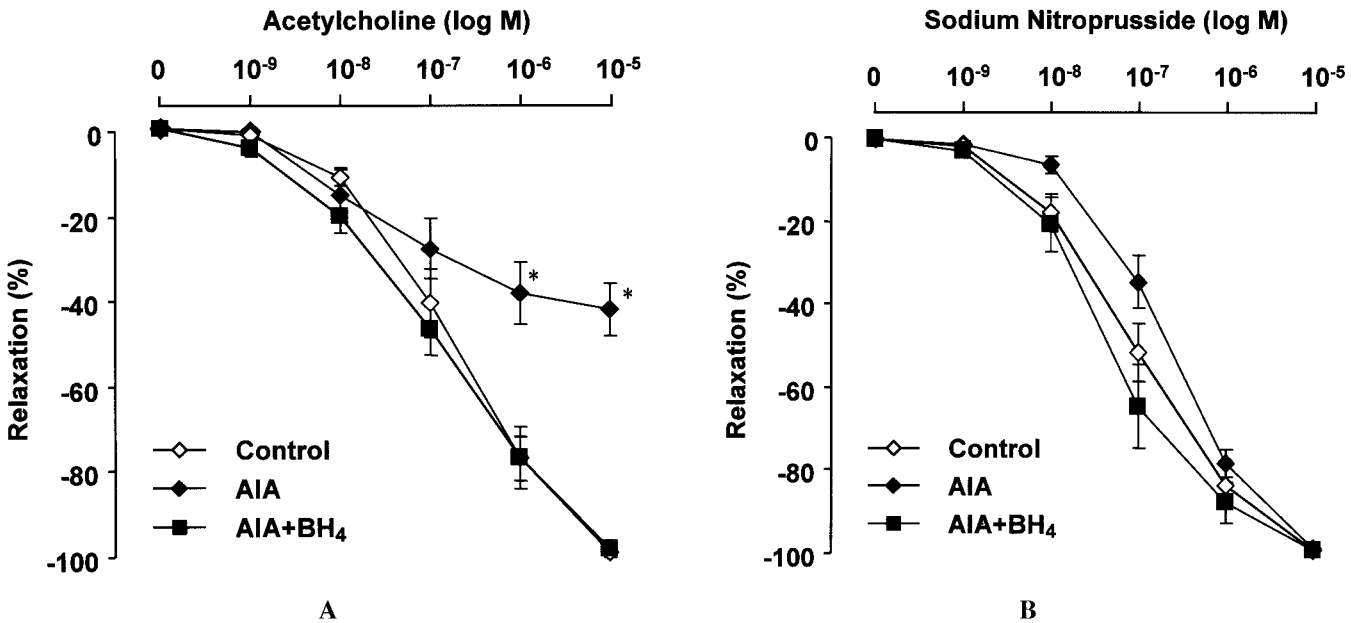

Figure 6. Treatment of adjuvant-induced arthritis (AIA) with tetrahydrobiopterin (BH_4) reverses endothelial dysfunction. BH_4 (20 mg/kg) was injected intraperitoneally into AIA rats from day 14 to day 20 after the onset of arthritis. On day 21, thoracic aortas were isolated from control and AIA rats that were untreated or treated with BH_4. After near-maximal contraction of the aortic ring with norepinephrine, dose responses to the indicated concentrations of **A,** acetylcholine (an endothelium-dependent vasodilator) or **B,** sodium nitroprusside (an endothelium-independent vasodilator) were determined. Relaxation was expressed as the percentage of the precontracted tension induced by norepinephrine. Values are the mean ± SEM (n = 6 rats per group). $* = P < 0.01$ versus control rats or versus AIA rats treated with BH_4.

Levels of NAD(P)H-stimulated O_2^- production are elevated in animals with hyperinsulinemia or hypercholesterolemia (26,27). Activity of NAD(P)H oxidases and levels of expression of p22phox, a subunit of the enzyme, are increased in aortas of hypertensive rats (28,29). Our results indicate that the common mechanism for the generation of O_2^-, which is seen in other atherogenic conditions, contributes at least in part to the pathophysiology of endothelial dysfunction caused by systemic inflammation.

What mediators are involved in the activation of vascular NAD(P)H oxidases in systemic arthritis? The activity and expression of vascular NAD(P)H oxidases are regulated by humoral factors and proinflammatory cytokines such as TNFα (23). TNFα is of particular interest, since the role of this cytokine has been well recognized in the disease process of RA and in arthritis models. We measured serum TNFα levels in AIA rats at the time the aortas were isolated. However, serum levels of TNFα at that point were below the sensitivity of the enzyme-linked immunosorbent assay used in our study (results not shown). Other inflammatory mediators might be involved in activation of the NAD(P)H oxidases. However, we cannot exclude the possibility that undetectable levels of TNFα may activate these vascular enzymes or that higher TNF levels may occur earlier in the course of AIA. Indeed, rapid improvement in endothelial dysfunction was reported in RA patients after infusion of anti-TNF antibodies (10).

We identified eNOS as another important source of vascular O_2^- excess in AIA. When homogenates of AIA rat aortas were incubated with L-arginine, the O_2^- production was significantly increased. This was in contrast to control rat aortas, in which addition of L-arginine decreased the O_2^- production. Endothelial NOS is a homodimeric enzyme that generates NO and L-citrulline from L-arginine. However, in certain circumstances, eNOS may cause uncoupling, which generates O_2^- (30). Uncoupled eNOS is reported to be a possible prominent source of endothelial O_2^- in hypertension or diabetes (16,31). When exposed to oxidant stress, including $ONOO^-$, or when deprived of its cofactor BH_4 or substrate L-arginine, eNOS generates O_2^- rather than NO (15,16,32). In apolipoprotein E–knockout mice, $ONOO^-$-mediated BH_4 oxidation is identified as a pathogenic cause of an eNOS uncoupling (17). Our study further demonstrates a decreased level of SDS-resistant eNOS dimer in the AIA rat aorta by use of

immunoblot analysis after low-temperature SDS-PAGE. The monomerization of eNOS has been linked to uncoupling of the enzyme (15), although the significance of this finding is still controversial (33).

We found that serum levels of BH_4 in AIA rats were significantly lower than those in control rats. Addition of BH_4 reversed the L-arginine–induced O_2^- increase in isolated AIA rat aortas. Furthermore, in vivo treatment of AIA with BH_4 reversed the endothelial dysfunction. These results suggest that the deficiency of BH_4 may contribute in part to formation of the uncoupled eNOS. The mechanism mediating the decreased BH_4 availability in AIA was still not elucidated, but it may be related to impaired synthesis or increased catabolism for oxidation by ROS such as $ONOO^-$ (16,17). Uncoupling of eNOS may not be the initial source of O_2^- production. However, it is likely that uncoupled eNOS accelerates the ongoing formation of oxygen radicals in the vasculature. We speculate that the increased ROS production by NAD(P)H oxidase results in eNOS uncoupling, leading to further amplification of oxidative stress in the vasculature in AIA.

Vascular eNOS expression is increased in AIA at the mRNA and protein levels. It was reported that H_2O_2, but not O_2^- or hydroxyl radicals, increases eNOS expression in aortic endothelial cells in vitro at the transcriptional level (34). Thus, the enhanced levels of eNOS expression in AIA aortas may reflect the overproduction of H_2O_2, which is derived from O_2^-. However, it should be emphasized that our study shows that the enhanced production of eNOS contributes to ROS production, not NO synthesis, in the vasculature of the arthritis model.

The present study demonstrates that increased O_2^- production probably contributes to the decreased NO release and to the impaired endothelium-dependent relaxation in animal models of RA. NAD(P)H oxidases and up-regulated, uncoupled eNOS account for the O_2^- overproduction. The current data in the arthritis model have striking similarities to our previous findings in experimental diabetic nephropathy. That study demonstrated excessive oxidative stress and reduced NO production in the glomeruli of diabetic rats (18). NAD(P)H oxidase and uncoupled eNOS were shown to contribute to the glomerular ROS production, mediated by the loss of BH_4 availability (18). These findings suggest that a variety of initiating processes (e.g., hyperglycemia or systemic inflammation) lead to downstream events that have in common an altered synthesis or bioavailability of NO and ROS in the vasculature. We propose that RA can be included among the human vascular diseases that potentially lead to vascular structural damage.

Our study has important implications for therapeutic strategies for endothelial dysfunction caused by systemic inflammation. Recent reports have suggested that angiotensin-converting enzyme (ACE) inhibition or statin treatment reduces the vascular activation of NAD(P)H oxidase. In cholesterol-fed animals, angiotensin receptor blockage inhibits NAD(P)H-dependent vascular O_2^- production and, in parallel, improves endothelial dysfunction (27). Statin inhibits angiotensin II–induced ROS production by vascular smooth muscle cells in vitro and reduces aortic O_2^- production in hypertensive rats (35). Similarly, ACE inhibition or a statin might be effective for improving endothelial dysfunction caused by systemic inflammation. Our model may be used as a tool to examine these therapeutic options. Such studies would provide a rationale for determining an effective approach to reducing cardiovascular mortality rates in patients with RA.

ACKNOWLEDGMENTS

We thank Drs. S. Fujimoto and T. Sasaki, Kawasaki Medical School, for insightful comments.

REFERENCES

1. Wolfe F, Mitchell DM, Sibley JT, Fries JF, Bloch DA, Williams CA, et al. The mortality of rheumatoid arthritis. Arthritis Rheum 1994;37:481–94.
2. Symmons DP, Jones MA, Scott DL, Prior P. Long term mortality outcome in patients with rheumatoid arthritis: early presenters continue to do well. J Rheumatol 1998;25:1072–7.
3. Del Rincon I, Williams K, Stern MP, Freeman GL, Escalante A. High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis Rheum 2001;44:2737–45.
4. Bergholm R, Leirisalo-Repo M, Vehkavaara S, Makimattila S, Taskinen MR, Yki-Jarvinen H. Impaired responsiveness to NO in newly diagnosed patients with rheumatoid arthritis. Arterioscler Thromb Vasc Biol 2002;22:1637–41.
5. Gonzalez-Juanatey C, Testa A, Garcia-Castelo A, Garcia-Porrua C, Llorca J, Vidan J, et al. HLA-DRB1 status affects endothelial function in treated patients with rheumatoid arthritis. Am J Med 2003;114:647–52.
6. Hurlimann D, Forster A, Noll G, Enseleit F, Chenevard R, Distler O, et al. Anti-tumor necrosis factor-α treatment improves endothelial function in patients with rheumatoid arthritis. Circulation 2002;106:2184–7.
7. Sattar N, McCarey DW, Capell H, Mclnnes IB. Explaining how high-grade systemic inflammation accelerates vascular risk in rheumatoid arthritis. Circulation 2003;108:2957–63.
8. Van Doornum S, McColl G, Wicks IP. Accelerated atherosclerosis: an extraarticular feature of rheumatoid arthritis? Arthritis Rheum 2002;46:862–73.
9. Kaplan MJ, McCune WJ. New evidence for vascular disease in patients with early rheumatoid arthritis. Lancet 2003;361:1068–9.
10. Gonzalez-Juanatey C, Testa A, Garcia-Castelo A, Garcia-Porrua

C, Llorca J, Gonzalez-Gay MA. Active but transient improvement of endothelial function in rheumatoid arthritis patients undergoing long-term treatment with anti–tumor necrosis factor α antibody. Arthritis Rheum 2004;51:447–50.
11. Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determinant in atherosclerosis? Circulation 2004;109: II27–33.
12. Higashi Y, Yoshizumi M. New methods to evaluate endothelial function: method for assessing endothelial function in humans using a strain-gauge plethysmography: nitric oxide-dependent and -independent vasodilation. J Pharmacol Sci 2003;93:399–404.
13. Landmesser U, Harrison DG. Oxidant stress as a marker for cardiovascular events: Ox marks the spot. Circulation 2001;104: 2638–40.
14. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840–4.
15. Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. J Clin Invest 2002;109:817–26.
16. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 2003;111:1201–9.
17. Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, et al. Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. Circulation 2001;103:1282–8.
18. Satoh M, Fujimoto S, Haruna Y, Arakawa S, Horike H, Komai N, et al. NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. Am J Physiol Renal Physiol 2005;288: F1144–52.
19. Satoh M, Kashihara N, Fujimoto S, Horike H, Tokura T, Namikoshi T, et al. A novel free radical scavenger, edarabone, protects against cisplatin-induced acute renal damage in vitro and in vivo. J Pharmacol Exp Ther 2003;305:1183–90.
20. Morita Y, Gupta R, Seidl KM, McDonagh KT, Fox DA. Cytokine production by dendritic cells genetically engineered to express IL-4: induction of Th2 responses and differential regulation of IL-12 and IL-23 synthesis. J Gene Med 2005;7:869–77.
21. Tani Y, Ohno T. Analysis of 6R- and 6S-tetrahydrobiopterin and other pterins by reversed-phase ion-pair liquid-chromatography with fluorimetric detection by post-column sodium nitrite oxidation. J Chromatogr 1993;617:249–55.
22. Mochizuki S, Sipkema P, Goto M, Hiramatsu O, Nakamoto H, Toyota E, et al. Exogenous NO suppresses flow-induced endothelium-derived NO production because of depletion of tetrahydrobiopterin. Am J Physiol Heart Circ Physiol 2005;288:H553–8.
23. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 2000;86: 494–501.
24. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation 2003;108:1912–6.
25. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. Am J Physiol Regul Integr Comp Physiol 2003;285:R277–97.
26. Kashiwagi A, Shinozaki K, Nishio Y, Maegawa H, Maeno Y, Kanazawa A, et al. Endothelium-specific activation of NAD(P)H oxidase in aortas of exogenously hyperinsulinemic rats. Am J Physiol 1999;277:E976–83.
27. Warnholtz A, Nickenig G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, et al. Increased NADH oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. Circulation 1999;99: 2027–33.
28. Zalba G, Beaumont FJ, Jose GS, Fortuno A, Fortuno M, Etayo JC, et al. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. Hypertension 2000;35:1055–61.
29. Hamilton CA, Brosnan MJ, McIntyre M, Graham D, Dominiczak AF. Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. Hypertension 2001;37:529–34.
30. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. J Biol Chem 2001;276:14533–6.
31. Mollnau H, Wendt M, Szocs K, Lassegue B, Schulz E, Oelze M, et al. Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. Circ Res 2002;90:E58–65.
32. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J Biol Chem 2003;278:22546–54.
33. Bauersachs J, Schafer A. Tetrahydrobiopterin and eNOS dimer/monomer ratio: a clue to eNOS uncoupling in diabetes? Cardiovasc Res 2005;65:768–9.
34. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ Res 2000;86:347–54.
35. Wassmann S, Laufs U, Baumer AT, Muller K, Konkol C, Sauer H, et al. Inhibition of geranylgeranylation reduces angiotensin II-mediated free radical production in vascular smooth muscle cells: involvement of angiotensin AT1 receptor expression and Rac1 GTPase. Mol Pharmacol 2001;59:646–54.