Exogenous NO suppresses flow-induced endothelium-derived NO production because of depletion of tetrahydrobiopterin

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Am J Physiol Heart Circ Physiol 288: H553–H558, 2005. First published October 7, 2004; doi:10.1152/ajpheart.00408.2004.—Exogenous nitric oxide (NO) suppresses endothelium-derived NO production. We were interested in determining whether this is also the case in flow-induced endothelium-derived NO production. If so, then is the mechanism because of intracellular depletion of tetrahydrobiopterin [BH4; a cofactor of NO synthase (NOS)]? which results in superoxide production by uncoupled NOS? Isolated canine femoral arteries were perfused with 100 μM S-nitroso-N-acetylpenicillamine (SNAP; an NO donor) and/or 64 μM BH4. Perfusion of SNAP suppressed flow-induced NO production, which was evaluated as a change in the slope of the linear relationship between perfusion rate and NO production rate (P < 0.02 vs. control; n = 7). Subsequent BH4 perfusion returned the slope to the control level. Concomitant perfusion of SNAP and BH4 retained the control-level NO production (n = 7). Concomitant perfusion of SNAP and 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron; 1 mM; a membrane-permeable superoxide scavenger) also retained the control-level NO production (n = 7), whereas perfusion of Tiron after SNAP could not return the NO production to the control level (P < 0.02 vs. control; n = 7). We also found a significant decrease in BH4 concentration in the endothelial cells after SNAP perfusion. In conclusion, these results indicate that exogenous NO suppresses the flow-induced, endothelium-derived NO production by superoxide released from uncoupled NOS because of intracellular BH4 depletion.

nitric oxide synthase; shear stress; superoxide; pterin; uncoupling

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FLOW-INDUCED SHEAR STRESS exerted on the luminal (endothelial) surface is one of the major physiological stimuli that activates nitric oxide (NO) synthase (NOS) in the vascular endothelial cells regulating numerous vascular functions, including smooth muscle tension (4). Recently, we observed a linear relationship between perfusion rate (wall shear stress) and endothelium-derived NO production from isolated canine femoral arteries (15), and we clarified involvement of hyaluronic acid glycosaminoglycans in the shear-sensing mechanism (17).

Basal and stimulated productions of endothelium-derived NO were reported to be under negative-feedback regulation by NO itself. Vasorelaxation of the isolated rat thoracic aortic rings by Nω-nitro-L-arginine methyl ester (l-NAME), an NOS inhibitor, was attenuated after 20-min treatment with S-nitroso-N-acetylpenicillamine (SNAP), an NO donor, suggesting inhibition of the basal NO release by exogenous NO (13). NO production from endothelial cells cultured on microcarrier beads in response to bradykinin and increased flow was attenuated after treatment with SNAP (2). However, the underlying mechanisms of the negative-feedback regulation are needed to be investigated further.

Recently, tetrahydrobiopterin (BH4), one of the obligatory cofactors for NOS activation, has been attracting considerable attention due to its key role in the regulation of activity of all three isoforms of NOS (29). More recent reports (5, 9, 21, 26) showed that a decrease in intracellular availability of BH4 is related to the dysfunction of NOS. The inhibitory action of NO on neural NOS was attenuated by the addition of a BH4-regenerating system (8). BH4 is also known to regulate superoxide production from NO through stabilization of O2 bound around NOS active site (25, 27). Accordingly, we hypothesized the involvement of BH4 in the effects of exogenous NO on changes in flow-induced NO production, i.e., attenuation of NOS activity (NOS uncoupling) due to BH4 depletion, resulting in superoxide released from uncoupled NOS. We thus studied the role of BH4 in the changes in flow-induced NO production before and after exposure to exogenous NO.

In the present study, we investigated 1) how exogenous NO affects the flow-induced, endothelium-derived (endogenous) NO production using isolated, perfused canine femoral arteries (by perfusing SNAP); 2) the possible involvement of decreased availability of BH4 (by perfusing exogenous BH4 and by measuring BH4 concentration in endothelial cells); and 3) superoxide produced from NOS in relation to BH4 depletion (by perfusing a membrane permeable superoxide scavenger 4,5-dihydroxy-1,3-benzene disulfonic acid, Tiron) in the effects of exogenous NO on endogenous flow-induced NO production.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Research Committee of Kawasaki Medical School and conform with the standards for use of laboratory animals established by the Institute of Laboratory Animal Resources, National Academy of Sciences.

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EXOGENOUS NO AND FLOW-INDUCED NO PRODUCTION

Chemicals. Modified Krebs-Henseleit bicarbonate buffer (in mM: 11 d-glucose, 1.2 MgSO₄, 12 KH₂PO₄, 4.7 KCl, 120 NaCl, 25 NaHCO₃, and 2.5 CaCl₂·2H₂O), SNAP, and Tiron were purchased from Sigma (St. Louis, MO). BH₄ was supplied by BioMol Research Laboratories (Plymouth Meeting, PA). SNAP, BH₄, and Tiron were dissolved in a Krebs-Henseleit buffer solution (Krebs buffer). All solutions were purged with a gas mixture of 20% O₂-5% CO₂-75% N₂ and were kept at 37 ± 0.5°C and pH 7.4.

Animals and Isolated Vessel Preparation

Isolated vessels were prepared by following the previously described method (15, 17). Adult mongrel dogs of either sex (n = 38; seven vessels for each of four protocols described below and five vessels for each of two protocols on intracellular BH₄ measurement), weighing 13–32 kg, were initially sedated with ketamine (200 mg im) and anesthetized with pentobarbital sodium (30 mg/kg iv). Animals were then ventilated with a respirator pump (3–5 liters O₂/min; model VS600, Instrumental Development, Pittsburg, PA). These dogs were used mainly for other experimental studies on coronary circulation, and we isolated femoral arteries for the present study before the coronary circulation experiments. A 6-cm-long segment of femoral artery (outer diameter 3–4.5 mm in situ) was isolated from each animal. Immediately after isolation, each artery was flushed with a heparinized saline solution, cannulated with a silicone tube through a plastic connector, and then placed in a bath containing Krebs buffer (37 ± 0.5°C) with the vessel length being kept to 6 cm (in vivo length). These vessels were studied in the passive state without a constrictor. The diameter was therefore constant throughout the experiment (confirmed by observation with a charge-coupled device camera attached to a microscope). Thus at the same flow rate there were no changes in shear rate in the different experimental settings, i.e., observed differences were due to the interventions.

Evaluation of NO Production Rate

Recently, we have reported (15, 17) a linear correlation between perfusion rate (wall shear stress) and NO production from isolated perfused canine femoral arteries. In the present study, the same experimental model was applied. Briefly, the flow-induced, endothelium-derived NO production rate was evaluated as the product of perfusion rate and concentration of nitrite in the effluent. Perfusion rate was determined by timed collection; the weight of the effluent collected in a vial was measured by an electronic balance. About 200 µl of the effluent solution were collected after 3 min of perfusion at each perfusion rate. Nitrite concentration in the effluent was measured by a nitrite-nitrate analyzer (ENO-20; Eicom, Kyoto, Japan). We used the slope that correlates the relation between perfusion rate and NO production rate as a measure of “NO production capacity” of each vessel (see Fig. 1). In preliminary studies, the NO production rate of an isolated canine femoral artery did not change significantly in three or four runs of perfusion, indicating no changes in the relation between perfusion rate and NO production rate during the experimental period of each vessel. This was a time-control experiment. Thus this means that negative feedback was not present when vessels were exposed to endogenous NO.

Experimental Protocols for SNAP and BH₄

**Protocol 1.** To investigate an effect of exogenous NO (SNAP) on flow-induced NO production and involvement of BH₄, each vessel was perfused (steady flow and single pass) with the following solutions consecutively (n = 7). First, a vessel was perfused with Krebs buffer at a wide range of perfusion rates to evaluate the control level of flow-induced NO production. Second, 100 µM SNAP was perfused for 20 min, following the procedures by Ma et al. (13), and was then washed out with Krebs buffer for 5 min. Flow-induced NO production was measured again by perfusing Krebs buffer at a wide range of perfusion rates. Third, 64 µM BH₄ was perfused for 20 min, and flow-induced NO production was measured by perfusing the BH₄ solution at a range of perfusion rates. The applied BH₄ concentration was adopted from the previous study (27). Finally, after washout of BH₄ with Krebs buffer for 5 min, flow-induced NO production was measured again by perfusing Krebs buffer at a wide range of perfusion rates.

**Protocol 2.** In a separate set of experiments, to further investigate possible involvement of intracellular BH₄ depletion during SNAP perfusion, each vessel was perfused with Krebs buffer at a range of perfusion rates before and after a 20-min perfusion of a mixture of 100 µM SNAP and 64 µM BH₄ with 5 min of washout (n = 7).

**Experimental Protocols for SNAP and Tiron**

**Protocol 3.** To investigate the role of superoxide production during perfusion of SNAP due to NOS uncoupling by BH₄ depletion and 2) efficacy of scavenging superoxide during SNAP perfusion, each vessel was perfused with Krebs buffer at a range of perfusion rates and then after 20 min perfusion of a mixture of 100 µM SNAP and 1 mM Tiron with 5 min of washout (n = 7).

**Protocol 4.** As a separate set of experiments, to investigate the efficacy of scavenging superoxide after perfusion of SNAP, each vessel was perfused with the following solutions consecutively (n = 7). First, a vessel was perfused with Krebs buffer at a wide range of perfusion rates to evaluate the control level flow-induced NO production. Second, 100 µM SNAP was perfused for 20 min and was then washed out with Krebs buffer for 5 min. Flow-induced NO production was measured again by perfusing Krebs buffer at a wide range of perfusion rates. Third, 1 mM Tiron was perfused for 20 min, and flow-induced NO production was measured by perfusing the Tiron solution at a range of perfusion rates. Finally, after washout of Tiron with Krebs buffer for 5 min, flow-induced NO production was measured again by perfusing Krebs buffer at a wide range of perfusion rates.

In all four protocols above, the perfusion rate was within the range between 0.8 and 127.6 ml/min. Calculated shear stress ranged from 0.002 to 1.097 Pa (0.02–10.97 dyn/cm²).

**Measurement of Intracellular BH₄ Concentration**

To confirm a decrease in BH₄ concentration in the endothelial cells after SNAP pretreatment, we measured BH₄ concentration in the endothelial cells by adopting the HPLC method developed by Tani and Ohno (22). First, we perfused the isolated vessels with the following solutions: 1) Krebs buffer (30 min) or 2) Krebs buffer (5 min), 100 µM SNAP (20 min), and Krebs buffer (5 min for washout) (n = 5, respectively). We then scraped the endothelial cells from the vessel wall and suspended the cells in a mixture of 0.1 M sodium phosphate buffer (pH 3.0) containing 5% (vol/vol) methanol, 3 mM sodium octylsulfate, 0.1 mM disodium EDTA, and 0.1 mM ascorbic acid (to prevent oxidation). The cells were then homogenized by a ultrasound homogenizer (U200S, IKA Labortechnik, Staufen, Germany), and the homogenized solution was mixed with a mixture of 0.5 M perchloric acid containing 0.1 M disodium EDTA and 0.1 M Na₂S₂O₃ for protein separation. After centrifugation (15,000 rpm for 10 min; model 1710, Kubota, Tokyo, Japan) and filtration (0.45-µm pore size; Millex-HV Filter Unit, Millipore, Billerica, MA), we measured BH₄ concentration by HPLC (22). Briefly, a postcolumn NaNO₂ oxidizer (U200S, IKA Labortechnik, Staufen, Germany), and the homogenized solution was mixed with a mixture of 0.5 M perchloric acid containing 0.1 M disodium EDTA and 0.1 M Na₂S₂O₃ for protein separation. After centrifugation (15,000 rpm for 10 min; model 1710, Kubota, Tokyo, Japan) and filtration (0.45-µm pore size; Millex-HV Filter Unit, Millipore, Billerica, MA), we measured BH₄ concentration by HPLC (22). Briefly, by postcolumn NaNO₂ oxidizer

**Data Analyses**

Linear regression and correlation analyses were applied to evaluate the slope of the linear relationship between perfusion rate and NO
production rate (see Fig. 1). Changes in flow-induced NO production rate were evaluated and expressed as changes in the slope (normalized by control; Figs. 2–5). All values are expressed as means ± SE. Statistical analyses for the absolute values of slopes were conducted by the Wilcoxon signed rank test. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

**Protocol 1**

A representative relationship between perfusion rate and NO production rate for one of the canine femoral arteries studied before and after SNAP perfusion is shown in Fig. 1. In each step, a linear relationship was observed between the perfusion rate and NO production rate ($r^2 = 0.93–0.99$). After 100 μM SNAP was perfused for 20 min, the flow-induced NO production rate was decreased to 74% of the control level. To investigate the possible involvement of intracellular BH4 availability, 64 μM BH4 was perfused for 20 min, and then flow-dependent NO production rate was evaluated by changing the perfusion rate of the BH4 solution. The flow-induced NO production rate during BH4 perfusion returned to the control level (103%). However, after washout of BH4, the flow-induced NO production rate during perfusion of Krebs buffer decreased (76%). Thus it is suggested that the downregulation can be temporarily reversed, but there is no protection of NO production capacity.

Figure 2 summarizes the slopes (normalized by control for clarity) of the linear correlation equations between the perfusion rate and NO production rate of seven vessels. After SNAP was perfused for 20 min, the slopes were significantly decreased ($P < 0.02$ vs. control). During BH4 perfusion, the slopes were returned to the control level [$P = \text{not significant (NS)}$ vs. control, $P < 0.02$ vs. after SNAP]. After washout of BH4, the slopes decreased again ($P < 0.02$ vs. control, $P = \text{NS}$ vs. after SNAP, $P < 0.02$ vs. during BH4).

**Protocol 2**

Concomitant perfusion of SNAP and BH4 retained the control level of the flow-induced, endothelium-derived NO production ($P = \text{NS}$ vs. control, $n = 7$, Fig. 3), suggesting decreased availability of intracellular BH4 after perfusion of SNAP alone.

**Protocol 3**

The above results suggested depletion of BH4 by perfusing SNAP. Thus, to investigate 1) the possibility of enhanced superoxide production due to NOS uncoupling by decreased intracellular BH4 level (26) and 2) the efficacy of simultaneously scavenging superoxide during SNAP perfusion, the cell-membrane permeable superoxide scavenger Tiron (1 mM) was perfused concomitantly with 100 μM SNAP. Figure 4 summarizes the normalized slopes before and after concomitant perfusion of SNAP and Tiron ($n = 7$). In sharp contrast to the results in Fig. 2 and in the similar manner to Fig. 3, the suppressive effect of SNAP on endogenous NO production rate was not observed ($P = \text{NS}$).

**Protocol 4**

Next, to investigate the efficacy of scavenging superoxide after perfusion of SNAP, 100 μM SNAP and 1 mM Tiron were perfused consecutively ($n = 7$). During and after perfusion of Tiron, the NO production level did not return to the control level ($P < 0.02$ control vs. during and after Tiron, Fig. 5).
indicating that during SNAP perfusion superoxide was released due to BH4 depletion and suggesting that scavenging superoxide after exogenous NO perfusion was not effective at least within the duration of the present study.

In preliminary studies, perfusion of SNAP solution after 5 h of preparation of the solution did not cause any changes in flow-induced NO production (data not shown), indicating that the remaining compounds after complete NO release did not cause any effect. Perfusion of BH4 or Tiron only did not affect the flow-induced, endothelium-derived NO production (data not shown).

Using an NO sensor (16), we directly measured NO release from SNAP and obtained 100 nM NO for 100 M SNAP. To confirm a decrease in intracellular BH4 level in the endothelial cells, we directly measured the concentration of BH4 in the scraped endothelial cells. After SNAP perfusion, intracellular BH4 concentration was significantly lower (110.4 ± 7.6 ng·ml·g tissue, n = 5) compared with the level without SNAP pretreatment (227.0 ± 18.2 ng·ml·g tissue, n = 5; P < 0.001).

**DISCUSSION**

In the present study, we found the suppression of the flow-induced, endothelium-derived NO production by exogenous NO (SNAP). The suggested mechanism is the superoxide production by uncoupled NOS due to intracellular BH4 depletion. This suppression could be prevented by supplementing BH4 or by protection against superoxide.

A strong suppressive effect of SNAP (100 M) on endogenous NO production was observed in the present study (Figs. 1, 2, and 5). In agreement with our study is that relaxation of downstream target arterial strips by the effluent from the upstream endothelial cells cultured on microcarrier beads was attenuated after a 15-min treatment with 1 M SNAP (2). L-NAME-induced vasoconstriction of rat aortic rings was significantly attenuated after 20 min treatment with SNAP in a dose-dependent manner (0.25–5 M SNAP) (13). Different susceptibility of each target tissue or cell as well as different experimental setup may explain the different dose dependency to SNAP. Although effective concentration of SNAP was different in each experiment, 15–20 min exposure to SNAP
caused significant suppression in endogenous NO production stimulated by both agonists and shear stress.

Treatment of cultured ovine fetal pulmonary arterial endothelial cells (FPFAEC) with 1 mM sodium nitroprusside (SNP) for 2–24 h significantly decreased endothelial cell NOS (ecNOS) activity but not ecNOS mRNA or protein levels (19). In contrast, ecNOS protein expression in cultured human coronary arterial endothelial cells was downregulated by 24 h of incubation with 100 μM SNAP, resulting in decreased NO production (28). The present study was finished within 3 h after vessel isolation, and thus downregulation of ecNOS expression and/or induction of inducible NOS were not likely to be involved (3, 7). It is thus considered that the present results reflected decreased ecNOS activity due to intracellular BH4 depletion.

The flow-induced NO production after concomitant perfusion of 1) SNAP and BH4 or 2) SNAP and Tiron was at the same level as the control runs (Figs. 3 and 4), clearly demonstrating involvement of superoxide in the suppression mechanisms in association with NOS uncoupling by decreased BH4 availability. Vasquez-Vivar et al. (27) studied the BH4-involved regulation mechanism of superoxide production from ecNOS and concluded that BH4 prevents dissociation of ferrous-dioxygen complex. The same group also reported that BH4 inhibits superoxide production from neuronal NOS (25). Both above and below the physiological concentrations, BH4 induced release of superoxide and decreased NO availability (14). In addition, BH4 directly reacts with superoxide (10, 27). In apolipoprotein E-deficient mice, increased superoxide production results in the formation of peroxynitrite, which strongly oxidizes BH4, leading to self-propagating condition for superoxide and peroxynitrite production (11). Recently, Shiraishi et al. (20) reported that incubation of cultured human umbilical vein endothelial cells with the NO donor (dl)l-(E)-4-ethyl-2-[(E)-hydroxyiminol]-5-nitro-3-hexanamide (NOR3) for 10 min significantly decreased the activity of GTP cyclohydrolase I (the key enzyme for BH4 synthesis) via cGMP-dependent mechanism, resulting in decreased intracellular BH4 concentration. It is thus conceivable that in the present study, relative intracellular availability of BH4 decreased during SNAP perfusion due to overuse of BH4 for 1) heme reduction, 2) direct reaction with superoxide, and 3) oxidation by peroxynitrite and also to suppression of the BH4 synthesis process (e.g., GTP cyclohydrolase I). This speculation is supported by the control level NO production during BH4 perfusion after SNAP (Figs. 1 and 2) and during concomitant perfusion of SNAP and BH4 (Fig. 3) or SNAP and Tiron (Fig. 4). Ma et al. (13) reported that concomitant administration of SNAP and SOD significantly augmented the negative-feedback regulation of basal NO release. This contradictory result may be due to cell-membrane impermeability of SOD (CuZn-SOD), whereas Tiron is membrane permeable. SOD scavenged only extracellular superoxide, increased intracellular availability of superoxide and maybe peroxynitrite, and enhanced the suppressive effect. In contrast, Tiron scavenges superoxide both outside and inside the cell, decreased superoxide around the active site of NOS, and thus decreased the suppressive effect.

The reason for the decrease in NO production after washout of BH4 is still unclear. After a longer waiting time, the BH4 synthesis process might have returned to a normal state and endogenous NO production might have regained a normal function. However, because of experimental limitations and possible effects of inducible NOS as described above, we did not study a longer time course. Possible reasons for the remaining effect are as follows: 1) the BH4 synthesis process (e.g., GTP cyclohydrolase I) and/or the recycle reaction between BH4 and BH2 (dihydrofolate reductase) remain to be attenuated, and 2) unlike endogenous NO that is produced by NOS, exogenous NO does not cause coordinated regulation of synthesis of other factors such as BH4 and NADPH and of intake of l-arginine. GTP cyclohydrolase I feedback regulatory protein may be also involved. Further studies are needed to elucidate the underlying mechanisms.

Intracellular translocation of ecNOS was observed by bradykinin stimulation, suggesting changes in NOS activity due to dissociation and association with caveolae (18). In contrast, SNP did not significantly alter the subcellular localization of ecNOS in ovine FPFAEC (19). NO donors (SNP and SNAP) reversibly dissociated caveolin-1 scaffold, suggesting modulation of the distance between elements of signaling cascades harbored in caveolae (12). Thus localization of ecNOS upon production and/or supply of an excessive amount of NO may lead in part to downregulation of ecNOS activity.

Whereas NO therapy with NO donors, nitroso compounds, NO gas, and others is becoming popular in the case of endothelial dysfunctions (1, 6), a combination of NO therapy, supplementation of BH4, or its related compound and/or stimulation of BH4 synthesis with substrates (GTP and sepiapterin) and/or antioxidative reagents would be a new therapeutic approach to maintain the physiological intracellular BH4 level. In fact, supplementation of BH4 has been proven to be clinically useful by the recent studies on hypercholesterolemic patients and chronic smokers (9, 21, 24). Supplementation of sepiapterin is also effective to restore atherosclerotic vessels to the normal endothelial function (23).

In conclusion, the flow-induced endogenous NO production from the isolated canine femoral arteries is suppressed significantly by exogenous NO. It is suggested that exogenous NO causes NOS uncoupling (superoxide release) by intracellular BH4 depletion.

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REFERENCES


