Impaired Modulation of Sympathetic Excitability by Nitric Oxide After Long-term Administration of Organic Nitrates in Pigs

Johannes Zanzinger, PhD; Jürgen Czachurski, BEng; Horst Seller, MD

Background—Endogenous nitric oxide (NO) reduces sympathetic vasoconstriction by attenuating neuronal excitability in the brain stem and inhibition of postganglionic neurotransmission. We studied whether this modulation of sympathetic circulatory control by NO may be altered during chronic administration of NO donor drugs in pigs.

Methods and Results—Nitrate tolerance was induced by oral administration of isosorbide dinitrate (ISDN, 4 mg/kg per day for 4 weeks) in eight pigs. Four of them were chronically instrumented for the measurement of mean arterial blood pressure and cardiac output in the conscious state. ISDN treatment caused hemodynamic tolerance to NO donors and significantly increased the hypotensive responses to pharmacologic ganglionic blockade in conscious pigs. In general anesthesia, ISDN-treated animals and age-matched controls (n=5) had similar baseline renal sympathetic nerve activity and in both groups neither inhibition of NO synthases (NOS) nor administration of NO donors to the brain stem by intracerebroventricular (ICV) infusions caused significant changes in baseline renal sympathetic nerve activity. However, whereas sympathoexcitatory responses to glutamate (0.5 mL, 0.1 mol/L, ICV) or electrical stimulation of somatic nerve afferents were significantly potentiated by central NOS inhibition and attenuated by NO donors in controls, these treatments no longer had significant effects in ISDN-treated pigs. Furthermore, reflex sympathetic activation in response to intravenous NO donor treatment was more pronounced in nitrate tolerant animals, which suggests loss of central sympathoinhibitory effects of NO. Subsequent histology on brain stem slices with NADPH-diaphorase as NOS marker revealed significant reduction of NOS density in ISDN-treated pigs.

Conclusions—Long-term administration of organic nitrates reduces the number of NO-producing neurons in the brain stem and causes loss of inhibitory effects of NO on sympathetic excitability. This component of tolerance to organic nitrates may be important in patients confronted frequently with sympathetic activation caused by mental and/or physical stressors. (Circulation. 1998;97:2352-2358.)

Key Words: nitric oxide □ brain □ nervous system

Endogenous NO regulates vascular tone by direct actions on smooth muscle and, in addition, to a significant extent by inhibition of sympathetic vasoconstrictor mechanisms. Organic nitrates that are known to act through the release of NO are widely used as vasodilator drugs. The efficacy of these drugs, however, can be severely impaired by the development of nitrate tolerance. Although it has been shown that NO release from organic nitrates is preserved during tolerance in vivo, reduced vasodilator effects of NO caused by enhanced endothelial superoxide production and endothelin-1 release may be causes of true vascular tolerance. In addition, neurohumoral activation and pseudotolerance associated with increased sympathetic activity have been proposed to contribute the phenomenon. Under physiologic conditions, NO inhibits sympathetic vasoconstrictor influences by both reducing the release of noradrenaline from postganglionic sympathetic fibers and by attenuation of neuronal sympathetic excitability within the medullary areas that regulate sympathetic outflow from the brain stem. Because impaired modulation of sympathetic functions by NO could contribute to nitrate tolerance, we studied whether long-term organic nitrate treatment affects basal and activated SNA in pigs and whether vasoconstrictor effects of SNA may be altered in nitrate tolerance in vivo. In addition, we studied the distribution of NOS within the lower brain stem in both control and nitrate-tolerant pigs by NADPH-diaphorase staining.

Methods

Long-term Experiments

Young farm pigs (n=4, 16 to 20 kg body wt) were sedated with ketamine (10 mg/kg IM) and anesthetized with pentobarbital (12 to 15 mg/kg IV). Anesthesia was maintained after intubation with isoflurane (1.0% to 1.5%) in the inspired air consisting of 2:1 N2O/O2. An aseptic left thoracotomy was performed through the fourth intercostal space under positive pressure respiration. The pulmonary artery was instrumented with an transient time ultrasonic...
Selected Abbreviations and Acronyms

BP = blood pressure  
CO = cardiac output  
HR = heart rate  
ICV = intracerebroventricular  
ISDN = isosorbide dinitrate  
L-NNA = nitro-l-arginine  
MAP = mean arterial pressure  
nNOS = neuronal NOS  
NO = nitric oxide  
NOS = NO synthase  
PBS = phosphate-buffered saline  
RSNA = renal sympathetic nerve activity  
RVLML = rostral ventrolateral medulla  
SNAP = S-nitroso-N-acetylpenicillamine  
TPR = total peripheral resistance

flow probe (Triton, ART2) for measurement of cardiac output and with a chronic catheter for infusion of drugs. For measurement of arterial BP, another catheter was implanted in the ascending aorta. Catheters and wires were tunneled subcutaneously to the dorsal neck. The pigs received antibiotics (cephalosporin 1 mg/kg) and analgesia (metamizol 50 mg/kg) for 1 week after surgery and were allowed to recover from surgery for at least 7 days. During the recovery period, the pigs were familiarized with the laboratory and trained to rest quietly in a straw filled cage for experimental periods of 1 hour. Measurements were subsequently made in conscious animals at least twice per week during continuous registration of all parameters. To monitor the development of nitrate tolerance, dose-response relations for the NO donor SNAP were performed by intravenous infusion after periods of registration of baseline hemodynamic parameters.

Throughout the study the pigs were fed with a standard diet (400 to 500 g/d of a cereal-based diet containing 12.6 MJ metabolizable energy/kg with 15.5% crude protein) and had free access to tap water. For induction of nitrate tolerance, pigs received 4 mg/kg per day ISDN with the diet. Two other groups of pigs were housed similarly but were not chronically instrumented. One group (n = 4) received ISDN at the same dosage and the other group (n = 5, control) was fed with a similar diet without drugs for the same duration. The mean weight gain throughout the study period was 428±39 g/d. All animals were studied in acute experiments (see below) subsequent to the chronic observations. The care of the pigs and the execution of the experimental protocol were supervised by an independent veterinarian in accordance with German laws and the animal welfare regulations of the University of Heidelberg.

Acute Experiments

Acute experiments were performed with similar general anesthesia as described for long-term experiments. For infusion of drugs and for measurement of blood pressure, catheters were placed into a femoral vein and artery (advanced in the abdominal aorta). Antibiotics (100 mg/kg ampicillin) were given to prevent possible influences of infections such as the induction of expression of inducible NOS by bacterial endotoxins. The pigs were paralyzed by 0.2 mg/kg per hour pancuronium bromide and artificially ventilated by a tracheal tube. End-tidal CO₂ was kept at normal levels by adjustment of ventilatory depth and rate. Arterial blood gases were monitored with a blood gas analyzer (AVL 990, AVL List) and maintained in the normal range by administration of sodium bicarbonate solutions or adjustment of ventilation. Rectal temperature was maintained at 38.5°C by a thermostatically controlled infrared lamp. For measurements of CO in animals that were not instrumented long term, a biluminal (right atrium, pulmonary artery) 5F Swan-Ganz thermodilution catheter (Baxter) was inserted through a jugular vein and advanced through the right ventricle in the A pulmonaryis under blood pressure control. In the other pigs CO was measured by the chronically implanted transient time flow probes (ART, Triton). The similarity of CO data has been checked in previous studies. For recording of RSNA, the left renal nerve was retroperitoneally exposed, placed on bipolar platinum electrodes, and kept in a mixture of petroleum jelly and paraffin oil. Neural signals were amplified (×20 000 to 50 000; Tektronix AM 502), filtered (2 to 3 kHz), and stored and analyzed with a CED 1401 interface connected to an 80486 PC computer. RSNA was full-wave rectified and then resistance-capacity integrated with a time constant between 7 to 10 ms. To activate somatosympathetic reflex responses, the left great sciatic nerve (N ischiadicus) was placed on bipolar platinum electrodes, embedded in petroleum jelly, and connected to an isolated stimulator (Digitimer). Stimulation was performed to produce reproducible submaximal excitatory effects on RSNA by using consecutive trains of 20-second length with 10 to 20 V, 30 Hz, and 0.5- to 1-ms pulse duration at intervals of 120 seconds.

Detection of NO Synthases Within the Brain Stem

Brain stems were removed from the skull and placed in 0.1 mol/L PBS (pH 7.4) containing 4% paraformaldehyde for at least 24 hours (at 4°C). After fixation, tissue from all animals was rinsed with pure PBS and placed in 15% sucrose containing PBS for cryoprotection. Cryotome sections were cut ∼30 µm thick, and the free-floating sections were stained as follows. They were incubated in PBS containing 0.75 mg/mL of reduced nicotinamide adenine dinucleotide phosphate (NADPH, Boehringer, Mannheim) and 0.375 mg/mL nitro blue tetrazolium at 37°C for 3 hours. After incubation, the sections were rinsed in PBS, put on gelatin-coated glass slides, dried in air, dehydrated, and coverslipped in Permount. Mapping of the brain stem was done in analogy to Berman’s atlas of the cat brain, and slices derived from similar rostrocaudal locations normalized to the overall size of the medulla were used for the study. The RVLML region was also functionally identified by microinjections of glutamate (500 nL, 0.5 mol/L) in three pigs in vivo, yielding maximal sympathoexcitatory responses on microinjections at the following distances relative to the obex: 4.5 to 5.0 mm lateral, 3.5 to 4.0 mm rostral, 5.0 to 6.0 mm deep (from the dorsal surface of the medulla). For statistical comparison of NOS density, NOS-positive cells on two slices (∼3.0 and 4.0 mm rostrally to the obex, respectively) were counted on a total area of 50 mm² per animal including both the ventrolateral and the dorsal medullary region. As similarly observed in a previous study on different laboratory animal species, variation of NOS density between regions was small. Therefore, these data were pooled. NOS density is expressed in number of NOS-positive neurons/mm² slice.

Drugs and Infusions

SNAP was from Alexis Chemicals. All other drugs were from Sigma. All drugs were dissolved in distilled water. For preparation of the final concentrations, the substances were further diluted in Ringer solution shortly before administration. For ICV-administration, a catheter was inserted into the cerebroventricular space from the dorsal surface of the medulla at the level of the obex and advanced to the ventral surface of the medulla. The position of the catheter was functionally verified by instantaneous excitatory sympathetic responses to injections (0.3 mL) of glutamate, which are finally integrated within the brain stem by presypathetic neurons within the RVLML. Central NOS inhibition was carried out by short-term infusion (within 5 minutes, 1 mL/min ICV) of L-NNA (0.3 mmol/L). Effects of exogenous NO were tested by short-term ICV-infusion of SNAP (100 µmol/L) after NOS inhibition. As a test for central sympathoexcitability, 0.5 mL of 0.1 mol/L glutamate was injected (ICV) subsequent to the above pretreatments or sham control.

Data Analysis

HR was derived from the BP signal. TPR was calculated as (MAP—CVP)/CO, where central venous pressure (CVP) was assumed to be 2 mm Hg when no measured data were available (during CO measurements with flow probes). RSNA was resistance-capacity integrated and measured in arbitrary units (aU). CO and TPR...
Baseline Sympathetic Activity and Hemodynamics in Normal (n=5) and Nitrate-Tolerant Pigs (n=8) During Acute Experiments With General Anesthesia

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Nitrate-Tolerant</th>
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</thead>
<tbody>
<tr>
<td>RSNA, arbitrary units</td>
<td>12.5±2.3</td>
<td>14.3±2.2</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>90.5±6.9</td>
<td>97.4±4.5</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>115±5</td>
<td>145±8*</td>
</tr>
<tr>
<td>CO, L/min</td>
<td>3.53±0.12</td>
<td>3.47±0.37</td>
</tr>
<tr>
<td>TPR, mm Hg/L per minute</td>
<td>25.6±3.2</td>
<td>29.9±3.5</td>
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Body weight did not differ between groups (normal, 32.4±3.2 kg; nitrate-tolerant, 36.2±4.6 kg). * indicates significance (P<0.05) vs normal animals.

impairment of the arterial vasodilatory effects of additional exogenous NO (SNAP, 30 to 300 μg/kg), as indicated by reduced changes in vascular resistance occurred already after the first week of ISDN treatment and was further enhanced until the fourth week of treatment. To get an estimate of the relevance of autonomic influences on blood pressure in awake animals, ganglionic neurotransmission was blocked by hexamethonium (0.5 mg/kg IV) in the four chronically instrumented pigs during control conditions and in the fourth week of ISDN treatment. Hexamethonium caused significantly less hypotension during control conditions (MAP fell from 92.1±3.1 to 80.4±4.5 mm Hg) than in the nitrate-tolerant state (99.2±2.9 versus 70.8±1.8 mm Hg).

Changes in Sympathetic Functions Associated With Nitrate Tolerance

A comparison of baseline hemodynamics and RSNA between control and ISDN-treated pigs under general anesthesia in acute experiments is given in the Table. Figure 3 shows original tracings of baseline hemodynamics and RSNA and responses to activation of somatosympathetic reflexes by electrical stimulation of the great sciatic nerve in a control and a nitrate-tolerant pig during similar general anesthesia. Baseline sympathetic activity was slightly higher in the tolerant pig, but comparable RSNA increases and hemodynamic responses to sciatic nerve stimulation could be evoked in both animals, which suggests maintained integrity of theses reflexes. A summary of the effects of reflex activation on MAP and RSNA for all animals and the effects of acute intracerebroventricular inhibition of NO synthesis or NO donor treatment in control and nitrate-tolerant animals is shown in Figure 4. In contrast to controls, ISDN-treated pigs no longer responded to the pharmacologic variation of NO availability within the brain stem. When, as shown in Figure 5, glutamate was injected intracerebroventricularly as a test for tonic excitation of medullary vasomotor neurons, the hypertensive effects were, albeit not significantly, greater in ISDN-treated pigs. The disappearance of sympathoinhibitory effects of NO after ISDN treatment was similarly observed.

Effects of Central NOS Inhibition on Hemodynamic and Sympathetic Responses to Systemic Administration of NO Donors

To test whether nitrate tolerance may alter the impact of centrally acting NO on sympathetic and/or hemodynamic

**Figure 1.** Baseline hemodynamic parameters during long-term ISDN treatment (4 mg/kg per day) in conscious pigs (n=4). Course of MAP (in mm Hg), HR (in bpm), and normalized CO (L/min/kg W^0.75) during the observation period of 5 weeks. *P<0.05 vs control values (week 0).

Measurements of MAP, CO, and HR obtained from conscious pigs throughout the study period are shown in Figure 1. Chronic organic nitrate treatment (4 mg/kg per day) had only small hemodynamic effects. While significant increases in HR occurred during the first week of ISDN treatment, the other parameters were not significantly affected. However, as shown in Figure 2, significant

**Figure 2.** Development of nitrate tolerance during long-term ISDN treatment as indicated by reduced dilator effects of exogenous NO in conscious pigs (n=4). Effects of intravenous administration of the NO donor SNAP (30 to 300 μg/kg) on normalized TPR (in mm Hg/L/min per kilogram W^0.75) are shown.
responses to systemically administered NO donors, SNAP (40 μg/kg) was given intravenously after NOS inhibition in the brain stem (ICV) in five control and four ISDN-treated pigs. The results are shown in Figure 6. Whereas SNAP caused significantly smaller decreases in blood pressure in nitrate-tolerant animals, the correspondent increases in sympathetic activity in response to baroreceptor unloading were even slightly greater than during control conditions.
NOS Density Within the Brain Stem

Figure 7A shows NADPH-positive structures within the brain stem at the level of the RVLM in a control and a nitrate-tolerant pig. A comparison of NOS density between control and nitrate-tolerant pigs is given in Figure 7B. Nitrate-tolerant pigs had significantly fewer NOS-positive neurons, which indicates that nitrate administration reduced NOS density by ∼50% in these animals.

Discussion

The major finding of this study is that long-term administration of exogenous NO in the form of organic nitrates may reduce endogenous NO synthesis within the brain stem and cause almost complete disappearance of the NO-mediated inhibition of sympathetic excitability observed in normal pigs. Such loss of sympathoinhibitory effects of both endogenous and exogenous NO after long-term nitrate administration may contribute to the clinical phenomenon of nitrate tolerance in patients receiving long-term nitrate therapy.

Impaired Inhibition of Sympathetic Excitability Versus Neurohumoral Activation

Our experiments suggest that the mechanisms underlying the observed changes in the modulation of sympathetic functions by NO during long-term nitrate treatment may be different from the so-called neurohumoral activation, which may be caused by stimulation of the renin-angiotensin system associated with blood volume expansion and increased circulating aldosterone.
levels in response to long-term treatment with organic nitrates.\textsuperscript{13,19,20} However, throughout the 4-week period of ISDN treatment in the pigs studied here, BP probably has always been higher than the threshold for significant activation of renin release. Moreover, increases in sympathetic activity that probably occurred in the animals are known to further increase this threshold.\textsuperscript{23} Furthermore, it has been recently shown in dogs\textsuperscript{23} and in patients\textsuperscript{23,24} that activation of the renin-angiotensin system may not be critically involved in nitrate tolerance. Nevertheless, the fact that heart rate was significantly increased during the first week of ISDN treatment could mean that some baroreflex-mediated increase in sympathetic activity may have initially contributed to the responses to ISDN.

Another factor contributing to nitrate tolerance, often called pseudotolerance,\textsuperscript{25} is the enhancement of sympathetic activity during long-term nitrate administration. Our results are largely in accordance with those of Stewart et al.,\textsuperscript{25} who similarly observed in conscious dogs much greater decreases in MAP in response to ganglionic blockade by hexamethonium in nitrate-tolerant animals, suggesting that overall sympathetic activity may be markedly higher during nitrate tolerance. These authors also reported that during anesthesia, vasodilator effects of NO donors became considerably greater in tolerant dogs because of the reduced sympathetic activity under these conditions, which is in accordance with the present findings. However, the term pseudotolerance probably does not correctly describe the mechanisms underlying activation of sympathetic activity in nitrate tolerance. Together with our previous observations,\textsuperscript{15} the results of this study instead suggest that nitrates cause specific impairment (tolerance) of the modulatory effects of NO on sympathoexcitatory afferents to the vasomotor center in the brain stem, that is, the RVLM. A term such as “sympathetic tolerance” therefore would be more appropriate to describe this phenomenon. Sympathoexcitation by afferents to the RVLM is most relevant. Sympathoexcitation by afferents to the vasomotor center in the brain stem, (tolerance) of the modulatory effects of NO on sympathoexcitation by NO. In other words, in nontolerant animals, NO activity during long-term nitrate administration. Our results contribute to the responses to ISDN.

**Perspectives**

We have shown that long-term administration of organic nitrates causes not only vascular tolerance but also a tolerance to the inhibitory effects of NO on sympathetic excitability. These functional alterations are associated with a reduction of NOS density within the brain stem. NO-induced downregulation of NOS observed in the present study could also occur during inflammatory or infectious diseases associated with expression of inducible NOS when endogenous storage forms of NO such
as S-nitrosothiols reach the brain through the bloodstream.\textsuperscript{12,33} Because sympathetic excitability of nitrate-tolerant pigs was considerably lower than that of normal pigs acutely treated (ICV) with NOS inhibitors, endogenous counterregulatory mechanisms may have partially replaced the functions of NO on central sympatoexcitatory neurons. It will be interesting to study these adaptations that prevent hypersensitivity to sympa-thoexcitatory stimuli. Another important question to be studied is to what extent current concepts for the prevention of nitrate tolerance, for example, intermittent therapy,\textsuperscript{34,35} new NO donor compounds,\textsuperscript{36} or cotreatment with antioxidants,\textsuperscript{11,37,38} prevents or reduces the structural and functional changes in sympathetic functions caused by long-term nitrate therapy.

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\textbf{References}

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