

Deactivation of Norepinephrine by Peroxynitrite as a New Pathogenesis in the Hypotension of Septic Shock

Ko Takakura, M.D., Ph.D.,* Wen Xiaohong, M.D.,† Kenji Takeuchi, M.D.,‡ Yoshikazu Yasuda, M.D., Ph.D.,‡ Satoru Fukuda, M.D., Ph.D.§

Background: Vascular hyporeactivity to catecholamines limits successful treatment of hypotension in septic shock. Large amounts of nitric oxide (NO) and superoxide anion (O_2^{-1}) are produced in response to bacterial endotoxins and/or inflammatory cytokines. NO reacts with O_2^{-1} to form the potentially toxic NO metabolite, peroxynitrite ($ONOO^{-1}$). The purpose of this study was to investigate whether $ONOO^{-1}$ decreases the vasoconstrictile activity of norepinephrine.

Methods: Norepinephrine was treated with $ONOO^{-1}$ or 3-morpholinonydonimine-*N*-ethyl-carbamate (SIN-1; an $ONOO^{-1}$ producer) in a 5×10^{-2} M sodium phosphate buffer solution at pH 7.4, and absorbance of the product was measured spectrophotometrically at 295 and 370 nm. Norepinephrine pretreated with $ONOO^{-1}$ was administered to isolated rat thoracic aortas to observe contractions in functional experiments. The rate constant between norepinephrine and $ONOO^{-1}$ was determined via a competition assay with cysteine in functional experiments. Norepinephrine pretreated with $ONOO^{-1}$ was injected intravenously into anesthetized rats to measure blood pressure.

Results: Norepinephrine pretreated with $ONOO^{-1}$ was confirmed spectrally as oxidized norepinephrine. Norepinephrine pretreated with $ONOO^{-1}$ decreased its vasoconstrictile force in an $ONOO^{-1}$ (10^{-6} , up to 3×10^{-4} M) concentration-dependent manner ($EC_{50} = 5.1 \times 10^{-5}$ M). The decrease in its force was lower at pretreatment with $ONOO^{-1}$ in a lower pH buffer. A rate constant for the $ONOO^{-1}$ -norepinephrine reaction was 6×10^2 M/s. Norepinephrine (10^{-7} M) incubated with SIN-1 (10^{-3} M) decreased its vasoconstrictile force in an incubation time-dependent manner. Administration of norepinephrine pretreated with $ONOO^{-1}$ to anesthetized rats caused no significant change in arterial blood pressure.

Conclusions: These results indicate that norepinephrine was oxidized and deactivated by $ONOO^{-1}$. This deactivation may, at least in part, account for the hyporeactivities of vasoconstriction to norepinephrine in septic shock.

VASCULAR hyporeactivity to catecholamines, such as dopamine, norepinephrine, and epinephrine, limits successful treatment of hypotension, a key feature in the pathophysiology of septic shock, leading to a high mortality rate. Although the precise mechanisms of vascular hyporeactivity have not been elucidated completely, large amounts of nitric oxide (NO) produced by the inducible isoform of NO synthase (iNOS) in response to bacterial endotoxins and/or inflammatory cytokines

seem to participate in the hyporeactivity.¹⁻³ iNOS has been identified in many tissues, including the vascular endothelium,⁴ smooth muscle,⁵ and myocardium.⁶

Recently it was reported that the exposure of norepinephrine to NO leads to 6-nitronorepinephrine,⁷ which has poor vasoactivities. Concomitant with the enhanced NO production, bacterial endotoxins or inflammatory cytokines also increase cellular superoxide anion (O_2^{-1}) production from xanthine oxidase, reduced nicotinamide adenine dinucleotide phosphate oxidase, mitochondria, arachidonic acid metabolism, constitutive NOS, and iNOS.^{8,9} O_2^{-1} also autoxidizes norepinephrine to an adrenochrome, which has poor vasoactivities.¹⁰ The poor vasoactivities of these derivatives nitrated by NO or oxidized by O_2^{-1} suggest their involvement in the pathogenesis of septic shock.

Nitric oxide reacts with O_2^{-1} at a nearly diffusion-limited rate, 6.7×10^9 M/s, to form the potentially toxic NO metabolite, peroxynitrite ($ONOO^{-1}$).¹¹ $ONOO^{-1}$ can also react with norepinephrine to form an oxidized derivative.¹² The second-order rate constant for the formation of $ONOO^{-1}$ is approximately three times greater than the rate of superoxide dismutase-catalyzed dismutation of O_2^{-1} , 2×10^9 M/s.¹³ Thus, $ONOO^{-1}$ formation is a favored reaction and occurs under conditions in which the cellular production of both NO and O_2^{-1} increases, such as in septic shock.^{14,15} Therefore, the oxidation of norepinephrine by $ONOO^{-1}$ may occur more favorably and may play a more important role in septic shock than nitration by NO or oxidation by O_2^{-1} . However, the vasoactivities of norepinephrine oxidized by $ONOO^{-1}$ have not been addressed either *in vitro* or *in vivo*.

Herein, we describe the vasoconstrictile effects of norepinephrine pretreated with $ONOO^{-1}$ or 3-morpholinonydonimine-*N*-ethyl-carbamate (SIN-1), which produces $ONOO^{-1}$ continuously, using isolated strips of rat thoracic aorta and anesthetized rats. If $ONOO^{-1}$ deactivates norepinephrine, this deactivation may account for the loss of vasoconstriction to norepinephrine in septic shock.

Materials and Methods

Animals

The protocols of this investigation were reviewed and approved by the institutional animal care committee of Fukui Medical University (Fukui, Japan). Male Wistar rats weighing 250–300 g were used.

* Assistant Professor, † Research Fellow, ‡ Instructor, § Professor.

Received from the Department of Anesthesiology and Reanimatology, Fukui Medical University, Fukui, Japan. Submitted for publication September 3, 2002. Accepted for publication December 9, 2002. Supported by grant No. C2-12671458 (to Dr. Takakura) from the Ministry of Education, Science, Sports, and Culture, Tokyo, Japan.

Address reprint requests to Dr. Takakura: Department of Anesthesiology and Reanimatology, Fukui Medical University, 23-3 Shimoaizuki, Matsuoka, Fukui 910-1193, Japan. Address electronic mail to: takakura@fmsrsa.fukui-med.ac.jp. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Treatment with Peroxynitrite

A stock solution (8×10^{-2} M ONOO⁻¹ in 3×10^{-1} N NaOH) was purchased from Dojindo Laboratories (Kumamoto, Japan). As alkaline conditions are required for ONOO⁻¹ stability, the stock solution was diluted to appropriate concentrations with 3×10^{-1} N NaOH. The ONOO⁻¹ was added to a 5×10^{-2} M sodium phosphate buffer solution containing norepinephrine at a pH of 7.4 and a temperature of 37°C. The half-life of ONOO⁻¹ at a pH of 7.4 is less than 1 s.¹¹ To avoid decomposition of ONOO⁻¹ before full mixing, reaction solutions in tubes were rapidly stirred during bolus ONOO⁻¹ additions. The final pHs of all reaction solutions were checked to ensure adequate buffering. To exclude the influence of possible decomposition products of ONOO⁻¹, such as nitrate or nitrite, decomposed ONOO⁻¹ was used as a control. Decomposed ONOO⁻¹ was made by incubation for 10 min in a buffer with a pH of 7.4.^{16,17}

Treatment with SIN-1

Norepinephrine (3×10^{-5} M) was incubated with 10^{-3} M SIN-1 in a 5×10^{-2} M sodium phosphate buffer solution at a pH of 7.4 and a temperature of 23°C for 0, 10, 30, 90, or 180 min. Under these conditions, SIN-1 produces submicromolar ONOO⁻¹ per minute for several hours continuously.¹⁸

Confirmation of Norepinephrine Oxidation

Norepinephrine oxidation was confirmed spectrophotometrically¹² with a Shimadzu UV-160A (accuracy, ± 0.005 absorbance; reproducibility, ± 0.002 absorbance; Shimadzu, Tokyo, Japan). Norepinephrine has a maximal absorbance at 280 nm at a pH of 7.4. However, once it is oxidized, norepinephrine has dual peaks of absorbance at 295 nm (2) and 370 nm (1) when an appropriate concentration is used as a base line. The numbers in parentheses indicate relative amounts of absorbance.

Functional Experiments

The rats were anesthetized by isoflurane inhalation and were killed by decapitation; the thoracic aortas were isolated for the functional experiments.¹⁹ The thoracic aorta was placed in Krebs Henseleit solution (10^{-3} M; NaCl, 118; KCl, 4.7; NaHCO₃, 25; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 2.5; and glucose, 10 M; pH, 7.4). Helical strips were carefully prepared under a dissecting microscope. To avoid the possible involvement of endothelium-derived relaxing factors in the mechanical response, the lack of relaxation to acetylcholine was confirmed after the endothelium was rubbed off with filter paper.¹⁹ Each strip was carefully suspended in an organ chamber containing 30 ml Krebs Henseleit solution bubbled with 95% O₂-5% CO₂ at 37°C, and the tension changes were recorded isometrically. A resting tension of 0.5 g was applied over a 1-h equilibration period.

As 0.1 ml norepinephrine pretreated with ONOO⁻¹ was added into the organ chamber containing 30 ml Krebs Henseleit solution, the final concentration of norepinephrine acting on the vascular strips was 1/300 of the norepinephrine pretreated with ONOO⁻¹. The final concentrations of norepinephrine in the Krebs Henseleit solution and the concentrations of ONOO⁻¹ in the reaction with norepinephrine in the sodium phosphate buffer are shown in the text and in the figures.

Contractions were expressed in terms of milligrams of contractile force developed divided by milligrams of wet tissue weight. Relaxations were expressed as a percentage of norepinephrine-induced contraction.

Determination of Rate Constants via Competition Assay

Because ONOO⁻¹ decomposes so rapidly, it is not possible to determine a deactivation rate constant by measuring norepinephrine activity over time. Therefore, bimolecular rate constants for reactions of ONOO⁻¹ and norepinephrine were determined by a competition assay, as previously described in detail.^{16,20} Based on the concentration response for ONOO⁻¹-mediated deactivation, an ONOO⁻¹ concentration of 5×10^{-5} M was chosen, which would inhibit more than 90% but less than 100% of 10^{-7} M norepinephrine-induced contraction so that protection by cysteine could be precisely measured. Norepinephrine solutions were prepared in a sodium phosphate buffer, and cysteine was added immediately before ONOO⁻¹. Remaining norepinephrine activity was determined by vasocontraction in a functional experiment. By comparing the ability of different cysteine concentrations to prevent ONOO⁻¹-mediated deactivation of the fixed concentration of norepinephrine, the rate constant for the ONOO⁻¹-norepinephrine reaction can be calculated on the basis of the following equation:

$$F[\text{norepinephrine}]/(1 - F)$$

$$k_{\text{cysteine}} = [\text{cysteine}]/k_{\text{norepinephrine}}$$

The terms F and k describe the fraction of norepinephrine deactivated by ONOO⁻¹ and the rate constant, respectively. Cysteine reacts with ONOO⁻¹ at a known rate (k_{cysteine}) of 5×10^3 M/s at a pH of 7.4 and a temperature of 37°C. When F[norepinephrine]/(1 - F) k_{cysteine} is plotted as a function of cysteine concentration, the reciprocal of the slope gives $k_{\text{norepinephrine}}$ in molar per second.

Measurement of Blood Pressure

During anesthesia with an intramuscular injection of pentobarbitone (30 mg/kg), a patent airway was established by tracheotomy and subsequent tracheal cannulation during spontaneous ventilation. The internal carotid artery and vein were cannulated to create a route for

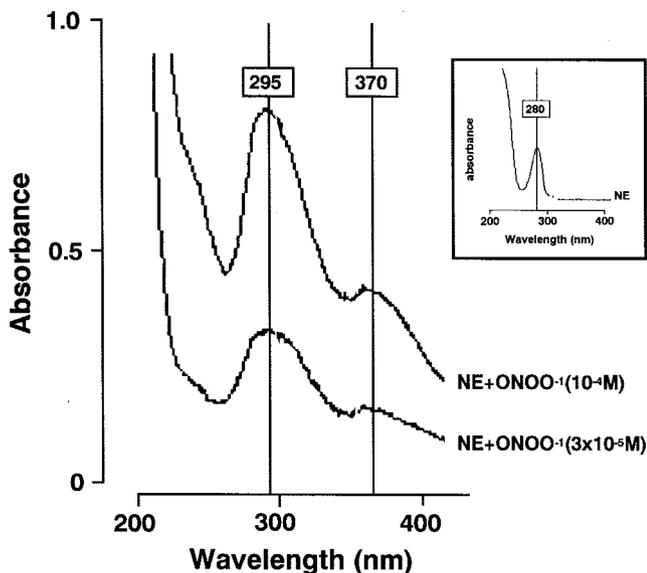


Fig. 1. The absorbance curves of norepinephrine (NE; 3×10^{-5} M) treated with peroxynitrite (ONOO^{-1}) measured spectrally. The inset shows the absorbance curve of norepinephrine.

continuous measurement of arterial pressure and a route for infusion, respectively.²¹ The infusion was maintained with physiologic saline (mixture of 3.4 mg/ml pentobarbitone for anesthetic maintenance) at a rate of $2.0 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. The rectal temperature was kept stable at 37°C by warm infusion and by laying pieces of aluminum foil over each rat. Drugs were administered intravenously by a microinjector, and each volume was 25–35 μl .

Chemicals

Peroxyntirite and SIN-1 were purchased from Dojindo Laboratories (Kumamoto, Japan), and the other drugs were purchased from Sigma (St. Louis, MO).

Statistical Analysis

Results are expressed as mean \pm SD. Group differences were analyzed by one-way analysis of variance and Scheffé F test as *post hoc* comparisons for multiple comparison at a significance level of 0.05. These analyses

were performed on a personal computer using Stat View II 4.0 software (Abacus Concepts, Berkeley, CA).

Results

Confirmation of Norepinephrine Oxidation

After treatment with ONOO^{-1} , the maximal absorbance of norepinephrine shifted from 280 (inset in fig. 1) to 295 nm (fig. 1). In addition, a new peak appeared at 370 nm. The rate of absorbance between 295 and 370 nm was approximately 2:1. The absorbances at 295 and 370 nm increased in a ONOO^{-1} concentration-dependent manner.

Contractile Force of Norepinephrine Pretreated with Peroxynitrite

Norepinephrine induced concentration-dependent contraction in endothelium-denuded aorta, and 10^{-7} M norepinephrine induced submaximal contraction. Norepinephrine pretreated with ONOO^{-1} decreased its contractile force in a ONOO^{-1} concentration-dependent manner ($\text{EC}_{50} = 5.1 \times 10^{-5}$ M; fig. 2A, solid circles). Representative tracings on contraction by norepinephrine pretreated with ONOO^{-1} are shown in figure 2B.

Conversely, norepinephrine pretreated with decomposed ONOO^{-1} (up to 3×10^{-4} M) did not decrease its contractile force (fig. 2A, open circles). An excessively high concentration of decomposed ONOO^{-1} decreased the contractile force of norepinephrine ($\text{EC}_{50} = 2.3 \times 10^{-3}$ M).

The decrease in the ONOO^{-1} -pretreated norepinephrine-induced contractile force was pH dependent (*i.e.*, the decrease reduced at pretreatment in a lower pH buffer; fig. 3).

To test the competition between oxidized and nonoxidized norepinephrine, after norepinephrine (10^{-7} M) pretreated with ONOO^{-1} (3×10^{-4} M), which had no contractile force (as shown in fig. 2), was added to the Krebs Henseleit solution in an organ chamber, norepinephrine was cumulatively applied. The contraction in-

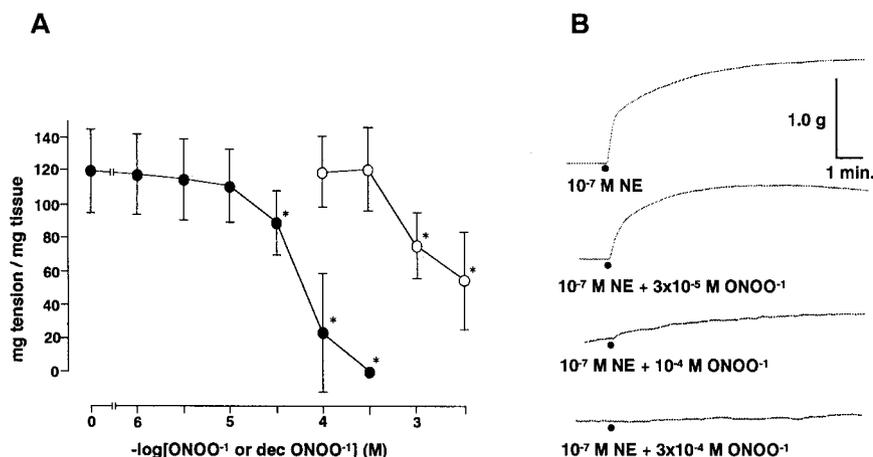


Fig. 2. (A) Contractile forces induced by norepinephrine pretreated with various concentrations of peroxynitrite (ONOO^{-1} ; solid circles) or decomposed ONOO^{-1} (open circles). After 0.1 ml norepinephrine (3×10^{-5} M) was added to an organ chamber containing 30 ml Krebs Henseleit solution, the final concentration of norepinephrine was 10^{-7} M. Data are expressed as mean \pm SD of eight experiments. $^*P < 0.05$ compared with no ONOO^{-1} treatment. (B) Representative tracings of the contractile forces induced by norepinephrine (NE) pretreated with various concentrations of ONOO^{-1} .

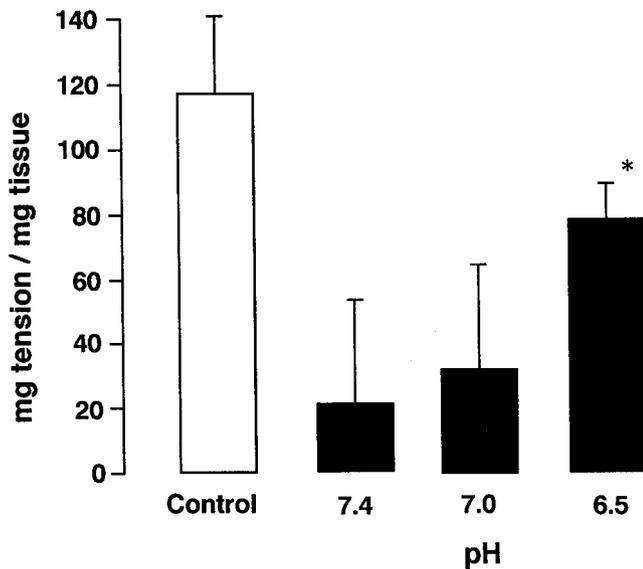


Fig. 3. The effects of sodium phosphate buffer pH on contractile forces induced by 3×10^{-5} M norepinephrine pretreated with 10^{-4} M peroxyntirite (ONOO^{-1}). After treated norepinephrine (0.1 ml) was added to an organ chamber containing 30 ml Krebs Henseleit solution, the final concentration of the norepinephrine was 10^{-7} M. Control: 3×10^{-5} M norepinephrine pretreated with 10^{-4} M decomposed ONOO^{-1} at pH 7.4. Data are expressed as mean \pm SD of eight experiments. There were significant statistical differences versus control ($P < 0.05$). * $P < 0.05$ compared with pH 7.4.

duced by norepinephrine was as much as that induced without norepinephrine pretreated with ONOO^{-1} (fig. 4).

Contractile Force of Norepinephrine Pretreated with SIN-1

Norepinephrine (10^{-7} M) incubated with SIN-1 (10^{-3} M) decreased its vasocontractile force in an incubation time-dependent manner (fig. 5). Ten-minute incubation with SIN-1 decreased the vasocontractile force of norepinephrine significantly, and 180-min incubation deactivated norepinephrine completely. The vasorelaxant activities of SIN-1 were stable (approximately 20% of norepinephrine induced contraction) for 180 min (data not shown).

Rate Constant for the Peroxynitrite-Norepinephrine Reaction

In a preliminary experiment, it was confirmed that cysteine pretreated with ONOO^{-1} had no effect on norepinephrine vasoactivities ($n = 5$). The rate reaction between ONOO^{-1} and norepinephrine was determined by measuring protection as a function of increasing free cysteine concentrations. By comparing the relative amounts of cysteine and norepinephrine and the known rate constant for the ONOO^{-1} -cysteine reaction, the rate constant for the ONOO^{-1} -norepinephrine reaction was determined to be 6×10^2 M/s (fig. 6).

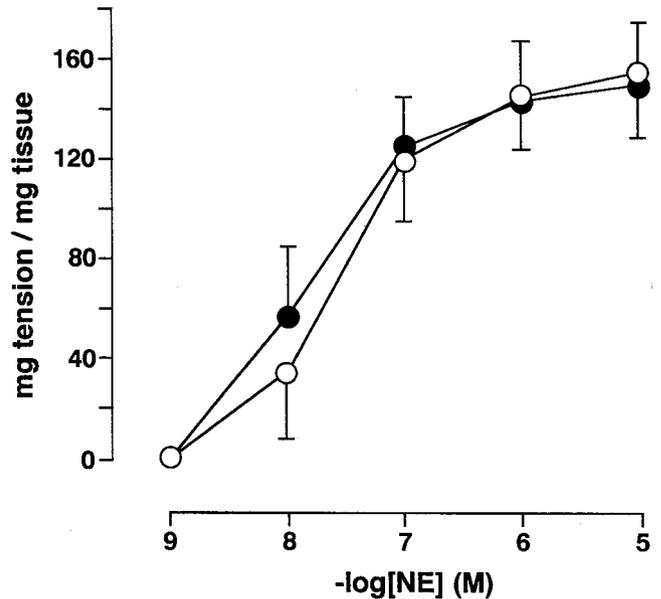


Fig. 4. The effects of norepinephrine (NE; 10^{-7} M) pretreated with peroxyntirite (ONOO^{-1} ; 3×10^{-4} M) to contractile forces induced by norepinephrine. Solid circles represent contractile forces induced by norepinephrine; open circles represent contractile forces induced by norepinephrine after the addition of norepinephrine (10^{-7} M) pretreated with ONOO^{-1} (3×10^{-4} M), which had no contractile force (see fig. 2). Data are expressed as mean \pm SD of eight experiments. There was no significant difference between groups.

Arterial Blood Pressure

Intravenous administration of $1 \mu\text{g}/\text{kg}$ norepinephrine increased systolic and diastolic arterial blood pressure significantly (table 1). The increases were prompt and transient. In contrast, administration of norepinephrine pretreated with ONOO^{-1} had no significant effect on arterial blood pressure for 1 h. Figure 7 shows the representative continuously monitored arterial blood pressure results.

Discussion

The maximal absorbance of norepinephrine shifted from 280 to 295 nm after treatment with ONOO^{-1} in a ONOO^{-1} concentration-dependent manner (fig. 1). In addition, a new, smaller peak appeared at 370 nm (the rate of absorbance between 295 and 370 nm was approximately 2:1). As these spectral characteristics corresponded with those of oxidized norepinephrine,¹² ONOO^{-1} certainly oxidized norepinephrine in this study. The activities of norepinephrine pretreated with ONOO^{-1} on vascular smooth muscle contractions (fig. 2) and on consequent increases of blood pressure (table 1) were decreased. During the pretreatment of norepinephrine with ONOO^{-1} , ONOO^{-1} oxidizes norepinephrine, and the rest of ONOO^{-1} decomposes in a sodium phosphate buffer.¹¹ Therefore, there are three possible mechanisms to explain the decrease in vasocontractile

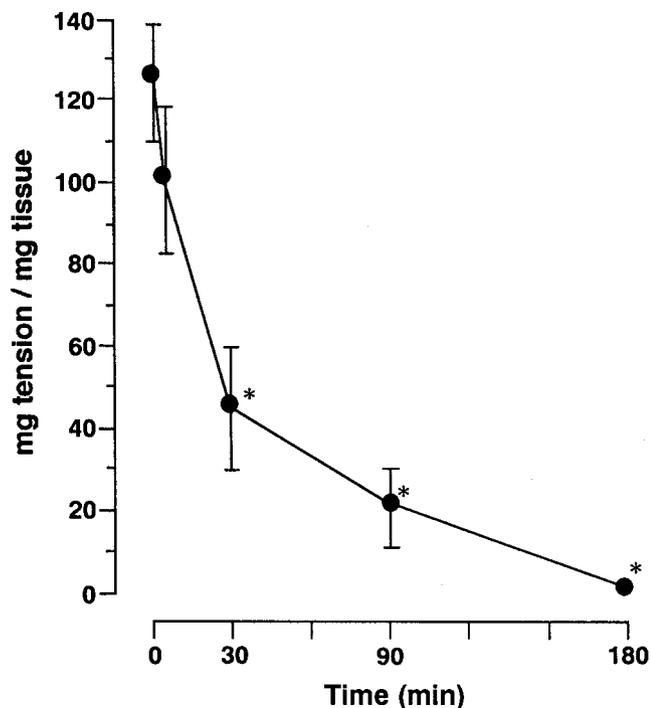


Fig. 5. Contractile force induced by norepinephrine incubated with 3-morpholinonydonimine-*N*-ethyl-carbamine (SIN-1). Norepinephrine was incubated with SIN-1 (10^{-3} M) in 5×10^{-2} M sodium phosphate buffer at 23°C. Data are expressed as mean \pm SD of eight experiments. * $P < 0.05$ compared with at 0 min incubation.

force of norepinephrine pretreated with ONOO^{-1} in our study: (1) oxidized norepinephrine does not induce vascular smooth muscle contraction, (2) decomposed ONOO^{-1} relaxes vascular smooth muscle and consequently inhibits norepinephrine-induced vasoconstriction because ONOO^{-1} or decomposed ONOO^{-1} stimulates cyclic GMP synthesis²² and relaxes²³ in vascular

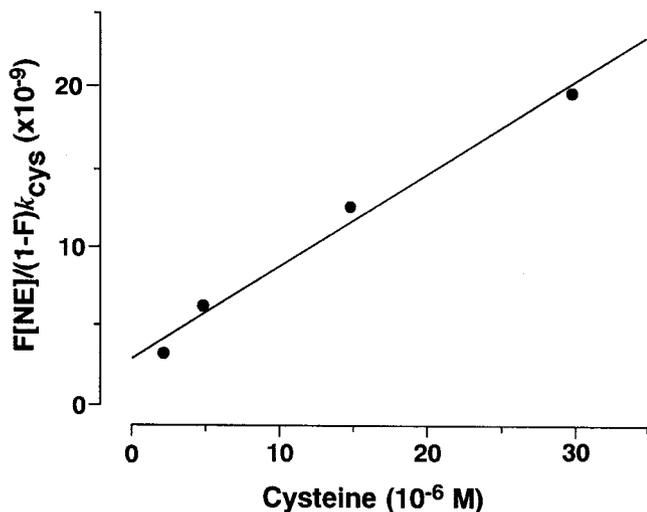


Fig. 6. Determination of a rate constant for the reaction of peroxynitrite (ONOO^{-1}) with norepinephrine (NE). Solid circles represent the mean of four experiments. F = the fraction of norepinephrine inactivated by ONOO^{-1} ; k = rate constant.

Table 1. Maximal Changes in Blood Pressure with Norepinephrine or Peroxynitrite-treated Norepinephrine

Treatment	Systolic Blood Pressure, mmHg	Diastolic Blood Pressure, mmHg
No treatment	105 \pm 7	91 \pm 10
Norepinephrine*	128 \pm 13§	106 \pm 5§
Peroxynitrite-treated norepinephrine†	105 \pm 5	91 \pm 4
Decomposed peroxynitrite-treated norepinephrine‡	125 \pm 11§	103 \pm 5§

Data are expressed as mean \pm SD of 6 experiments.

* 1 $\mu\text{g}/\text{kg}$ norepinephrine was injected. † 1 $\mu\text{g}/\text{kg}$ norepinephrine pretreated with 10^{-4} M peroxynitrite was injected. ‡ 1 $\mu\text{g}/\text{kg}$ norepinephrine pre-treated with 10^{-4} M decomposed peroxynitrite was injected. § $P < 0.05$ vs. no treatment.

smooth muscle, and (3) both (1) and (2). Norepinephrine pretreated with 3×10^{-4} M decomposed ONOO^{-1} did not decrease its vasoconstrictive activity (fig. 2), and the same concentration of ONOO^{-1} deactivated norepinephrine completely. Therefore, it was suggested that (1) is the most likely mechanism for the decrease in vasoconstrictive force of norepinephrine pretreated with ONOO^{-1} .

As norepinephrine (10^{-7} M) pretreated with ONOO^{-1} (3×10^{-4} M) had no contractile force, as shown in figure 2, it is believed that almost all of the norepinephrine was oxidized norepinephrine. During coexistence of the oxidized norepinephrine, norepinephrine-induced contractions were not inhibited (fig. 4). Therefore, oxidized norepinephrine does not have enough affinity to adrenoceptors to inhibit normal norepinephrine binding to adrenoceptors competitively.

It is well known that vascular hyporeactivity to catecholamines, such as dopamine, norepinephrine, and epinephrine, occurs in septic shock. Although the precise mechanisms of vascular hyporeactivity have not been elucidated completely, large amounts of NO produced by iNOS in response to bacterial endotoxins and/or inflammatory cytokines seem to participate in this hyporeactivity.¹⁻³ Recently it was reported that the exposure of norepinephrine to NO or NO-related compounds leads to 6-nitronorepinephrine,⁷ which has poor vasoactivities. Norepinephrine was almost completely

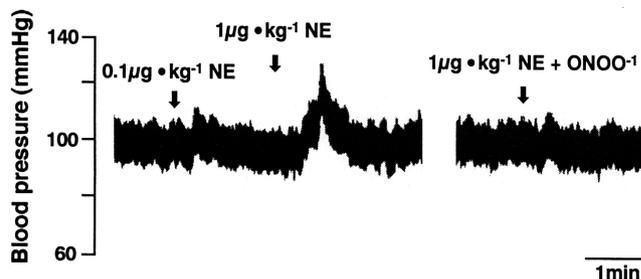


Fig. 7. Arterial blood pressure monitored continuously during intravenous administration of 1 $\mu\text{g}/\text{kg}$ norepinephrine (NE; left) or norepinephrine pretreated with 10^{-4} M peroxynitrite (ONOO^{-1} ; right).

Table 2. Rate Constants for Reactions with Peroxynitrite at pH 7.4

Substrate	<i>k</i> (m/s)
Norepinephrine	6×10^2
Cysteine*	5×10^3
Albumin*	4×10^3
Glutathione*	1×10^3

* Data from Ronson *et al.*²⁷

converted by NO or NO-related compounds to 6-nitronorepinephrine, not to oxidized norepinephrine. This nitration reaction requires extremely high NO concentrations (at least $> 10^{-2}$ M) and is very slow (rate constant, < 0.1 M/s).⁷ Therefore, the reaction hardly occurs under normal physiologic conditions, and it may not be a predominant reaction even in sepsis, in which the concentration of NO is micromolar.²⁴ In fact, although 6-nitronorepinephrine was identified in rat brain, the concentration was very low (4×10^{-8} M).²⁵ Conversely, multiple experiments have demonstrated that NO becomes highly reactive when converted to secondary reactive nitrogen species such as ONOO⁻¹.²⁶ Also in this study, ONOO⁻¹ reacted with and deactivated norepinephrine at a much faster rate (6×10^2 M/s) and at a much lower concentration (3×10^{-5} M) than NO. These results suggest that the oxidation of norepinephrine by ONOO⁻¹ may be a more predominant reaction than the nitration of norepinephrine by NO in sepsis.

The rate constant for ONOO⁻¹ and norepinephrine is not fast compared with that of other important antioxidants that react with ONOO⁻¹ (table 2).²⁷ However, the speed of the reaction is in proportion not only to the rate constant but also to the concentration of the reactant with ONOO⁻¹. Large amounts of other reactive oxygen species are produced, and the consequent loss of antioxidant concentrations occurs in septic shock.²⁸ Conversely, the concentration of norepinephrine increases because the endogenous norepinephrine released increases in shock, and exogenous norepinephrine is administered in the treatment of hypotension. Therefore, it is possible that ONOO⁻¹ might react with norepinephrine, as well as with antioxidants, in septic shock.

It has been suggested that the net exposure to ONOO⁻¹ should be shown as the area under the curve of time *versus* concentration.²⁹ Therefore, even low concentrations of ONOO⁻¹ may be able to deactivate norepinephrine during long-term incubation. Thus, norepinephrine was incubated with an ONOO⁻¹ producer, SIN-1. This drug releases both a few micromolars of NO and O₂⁻¹. continuously and, consequently, ONOO⁻¹.³⁰ One millimolar of SIN-1 released submicromolar ONOO⁻¹ continuously in sodium phosphate buffer solution at a pH of 7.4 and a temperature of 23°C¹⁸ and decreased the vasocontractile force of norepinephrine in an incubation time-dependent manner (fig. 5). SIN-1

also relaxes vascular smooth muscle, but the vasorelaxant activities were stable during this study. Therefore, it is believed that the time-dependent decrease in vasocontractile force of norepinephrine must be *via* the deactivation of norepinephrine by ONOO⁻¹ gradually released from SIN-1. Although ONOO⁻¹ is produced from stimulated vascular smooth muscles³¹ and endothelial cells,³² it is not known how much ONOO⁻¹ is produced in the vascular system environment during sepsis. However, it may be noteworthy that leukocytes accelerated to adhere to vascular endothelium by endotoxin can generate submicromolar ONOO⁻¹,³³ and the ONOO⁻¹ continuously generated during sepsis may be enough to deactivate norepinephrine.

Lower pH shortens the half-life of ONOO⁻¹.³³ This may explain why the contraction by norepinephrine pretreated with ONOO⁻¹ at lower pH was stronger than that at higher pH (fig. 3). It is well known that lactic acidosis is frequently present in septic shock patients due to decreased blood flow to visceral organs, as well as impaired oxygen extraction.³⁴ The correction of pH by alkaline salts may put patients at risk for deactivation of norepinephrine in the presence of ONOO⁻¹.

Oxidized norepinephrine administered intravenously had no effect on blood pressure (fig. 7, table 1). This result shows that oxidized norepinephrine cannot be reduced to norepinephrine even by the various antioxidants existing *in vivo*, such as glutathione, cysteine, vitamin C, and vitamin E—*i.e.*, the reaction between ONOO⁻¹ and norepinephrine was irreversible *in vivo*. If the reaction was reversible and the reduction of oxidized norepinephrine to norepinephrine occurred, the norepinephrine must have increased arterial blood pressure.

In conclusion, norepinephrine was oxidized and deactivated by ONOO⁻¹ at a rate constant of 6×10^2 M/s irreversibly in a pH-dependent manner. These results may, at least in part, account for the loss of vasoconstrictor tone to norepinephrine in septic shock.

The authors thank Joseph S. Beckman, Ph.D. (Professor, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon), for beneficial advice on ONOO⁻¹ treatments.

References

1. Stoclet JC, Muller B, Gyorgy K, Andriantsiohaina R, Kleschyov AL: The inducible nitric oxide synthase in vascular and cardiac tissue. *Eur J Pharmacol* 1999; 375:139-55
2. Gomez-Jimez J, Salgado A, Mourelle M, Martin MC, Segura RM, Peracaula R, Moncada S: L-arginine: Nitric oxide pathway in endotoxemia and human septic shock. *Crit Care Med* 1995; 23:253-8
3. de Werra I, Jaccard C, Corradin SB, Chioloro R, Yersin B, Gallati H, Assicot M, Bohuon C, Baumgartner J-D, Glauser MP, Heumann D: Cytokines, nitrite/nitrate, soluble tumor necrosis factor receptors, and procalcitonin concentrations: Comparisons in patients with septic shock, cardiogenic shock and bacterial pneumonia. *Crit Care Med* 1997; 25:607-13
4. Radomski, MW, Palmer RMJ, Moncada S: Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci U S A* 1990; 87:10043-7
5. Fleming I, Gray GA, Schott C, Stoclet J-C: Inducible but not constitutive

- production of nitric oxide by smooth muscle cells. *Eur J Pharmacol* 1991; 200:375-6
6. Schulz R, Nava E, Moncada S: Induction and potential biological relevance of a Ca^{2+} -independent nitric oxide synthase in the myocardium. *Br J Pharmacol* 1992; 105:575-80
 7. Nakaki T, Fujii T, Suzuki E, Shinatani F: Endothelium-induced and -dependent vasoactivity of 6-nitronorepinephrine. *Eur J Pharmacol* 1998; 357:193-7
 8. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM: Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* 1992; 267:24173-6
 9. Xia Y, Zweier JL: Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci U S A* 1997; 94:6954-8
 10. MacArthur H, Westfall TC, Riley DP, Misko TP, Salvemini D: Inactivation of catecholamines by superoxide gives new insights on the pathogenesis of septic shock. *Proc Natl Acad Sci U S A* 2000; 97:9753-8
 11. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990; 87:1620-4
 12. Daveu C, Servy C, Dendane M, Marin P, Ducrocq C: Oxidation and nitration of catecholamines by nitrogen oxides derived from nitric oxide. *Nitric Oxide* 1997; 1:234-43
 13. Boissinot M, Kuhn LA, Lee P, Fisher CL, Wang Y, Hallewell RA, Tainer JA: Rational design and expression of a heparin-targeted human superoxide dismutase. *Biochem Biophys Res Commun* 1993; 190:250-6
 14. Fukuyama N, Takebayashi Y, Hida M, Ishida H, Ichimori K, Nakazawa H: Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. *Free Radic Biol Med* 1997; 22:771-4
 15. Strand OA, Leone A, Giercksky KE, Kirkeboen KA: Nitric oxide indices in human septic shock. *Crit Care Med* 2000; 28:2779-85
 16. Takakura K, Beckman JS, MacMillan-Crow LA, Crow JP: Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite. *Arch Biochem Biophys* 1999; 369:197-207
 17. Takakura K, Taniguchi T, Muramatsu I, Takeuchi K, Fukuda S: Modification of α_1 -adrenoceptors by peroxynitrite as a possible mechanism of systemic hypotension in sepsis. *Crit Care Med* 2002; 30:894-9
 18. Crow JP, Beckman JS, McCord JM: Sensitivity of the essential zinc-thiolate moiety of yeast alcohol dehydrogenase to hypochlorite and peroxynitrite. *Biochemistry* 1995; 34:3544-52
 19. Takakura K, Goto Y, Kigoshi S, Muramatsu I: Comparison between the effects of treatment *in vitro* and *in vivo* with lipopolysaccharide on responsiveness of rat thoracic aorta. *Circ Shock* 1994; 42:141-6
 20. Radi R: Kinetic analysis of reactivity of peroxynitrite with biomolecules. *Methods Enzymol* 1996; 269:354-66
 21. Takakura K, Sugiura Y, Goto Y: Differential microcirculation dynamics during deliberate hypotension induced by nicardipine, PGE_1 , and trimethaphan in rat mesentery. *Can J Anaesth* 1995; 42:1035-9
 22. Tarpey MM, Beckman JS, Ischiropoulos H, Gore JZ, Brock TA: Peroxynitrite stimulates vascular smooth muscle cell cyclic GMP synthesis. *FEBS Lett* 1995; 364:314-8
 23. Liu S, Beckman JS, Ku DD: Peroxynitrite, a product of superoxide and nitric oxide, produces coronary vasorelaxation in dogs. *J Pharmacol Exp Ther* 1994; 268:1114-21
 24. Vinten-Johansen J: Physiological effects of peroxynitrite. *Circ Res* 2000; 87:170-2
 25. Shintani F, Kinoshita T, Kanba S, Ishikawa T, Suzuki E, Sasakawa N, Kato R, Asai M, Nakaki T: Bioactive 6-nitronorepinephrine identified in mammalian brain. *J Biol Chem* 1996; 271:13561-5
 26. Beckman JS, Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am J Physiol* 1996; 271:C1424-37
 27. Ronson RS, Nakamura M, Vinten-Johansen J: The cardiovascular effects and implications of peroxynitrite. *Cardiovas Res* 1999; 44:47-59
 28. Tsai K, Hsu T-G, Kong C-W, Lin K-C, Lu F-J: Is the endogenous peroxyl-radical scavenging capacity of plasma protective in systemic inflammatory disorders in humans? *Free Radic Biol Med* 2000; 28:926-33
 29. Beckman JS: Parsing the effects of nitric oxide, S-nitrosothiols, and peroxynitrite on inducible nitric oxide synthase-dependent cardiac myocyte apoptosis. *Circ Res* 1999; 85:870-1
 30. Hogg N, Darley-Usmer VM, Wilson MT, Moncada S: Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J* 1992; 281:419-4
 31. Boota A, Zar H, Kim YM, Johnson B, Pitt B, Davies P: IL-1 beta stimulates superoxide and delayed peroxynitrite production by pulmonary vascular smooth muscle cells. *Am J Physiol* 1996; 271:L932-8
 32. Kooy NW, Royall JA: Agonist-induced peroxynitrite production from endothelial cells. *Arch Biochem Biophys* 1994; 310:352-9
 33. Gagnon C, Leblond FA, Filep JG: Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide. *FEBS Lett* 1998; 431:107-10
 34. Nogare ARD: Southwestern internal medicine conference: Septic shock. *Am J Med Sci* 1991; 302:50-65