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Ketamine and Midazolam Differentially Inhibit Nonadrenergic Noncholinergic Lower Esophageal Sphincter Relaxation in Rabbits

Role of Superoxide Anion and Nitric Oxide Synthase

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Background: **The authors previously reported that ketamine and midazolam inhibited nitric oxide-mediated nonadrenergic noncholinergic (NANC) lower esophageal sphincter (LES) relaxation *via* nitric oxide-3',5'-cyclic guanosine monophosphate pathway modulation. The mechanisms inhibiting the NANC relaxation by ketamine and midazolam were investigated.**

Methods: **The isometric tension of circular distal esophageal muscle strips from Japanese White rabbits was examined. NANC relaxation was induced by KCl (30 mM) in the presence of atropine (3×10^{-6} M) and guanethidine (3×10^{-6} M). Nitric oxide synthase activity in the absence and presence of ketamine and midazolam was analyzed using the biochemical conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline.**

Results: **The ketamine-induced inhibition of the NANC relaxation was partly reversed by superoxide dismutase (200, 400 U/ml) but not by catalase (100 U/ml). Ketamine concentration-dependently inhibited the relaxation induced by N-ethyl-ethanamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine (diethylamine NONOate) and S-nitrosoglutathione. The NANC relaxation itself was not affected by superoxide dismutase. The midazolam-induced inhibition of the NANC relaxation was reversed neither by superoxide dismutase nor by catalase, and midazolam did not affect the relaxations induced by nitric oxide donors. The nitric oxide synthase activity was concentration-dependently suppressed by midazolam, but there was no marked effect of ketamine. Pyrogallol, a superoxide generator, inhibited the NANC and the diethylamine NONOate-induced relaxations. The pyrogallol-induced inhibition of the NANC relaxation was reversed by superoxide dismutase.**

Conclusion: **These findings suggest that ketamine inhibits NANC LES relaxation by the extracellular production of superoxide anion, and that midazolam inhibits it by the inhibition of nitric oxide synthase activity.**

The lower esophageal sphincter (LES) is an important specialized smooth muscle in the gastrointestinal tract for anesthesiologists because LES contractility is one of the crucial factors in preventing regurgitation during general anesthesia.[1] The enteric nervous system, similar to the extrinsic adrenergic and cholinergic innervations, is known to play a substantial role in mediating gastrointestinal motility by releasing various hormones and neurotransmitters.[2] Nonadrenergic noncholinergic (NANC) nerves mediate the peristaltic waves and the relaxing mechanisms of the gastrointestinal tract, including the LES.[3,4]

We previously reported that the NANC LES relaxation was mediated by nitric oxide (NO) or NO-related substances endogenously released from the myenteric plexus, and by K^+ channels of smooth muscle.[5] An example of actual tension recordings of the NANC relaxation is shown in figure 1A. In that investigation, ketamine and midazolam concentration-dependently inhibited the NANC relaxation, which is NO synthase dependent, and decreased the concentration of 3',5'-cyclic guanosine monophosphate (cGMP); however, these intravenous anesthetics did not alter the sodium nitroprusside (SNP)-induced relaxation, which is NO synthase independent.[5] These results suggest that ketamine and midazolam inhibit NANC relaxation *via* NO–cGMP pathway modulation, and they may possibly act on the synthesis, release, or transport of NO (Fig. 1B). In animal LES, neuronal NO synthase has been demonstrated in the myenteric plexus or submucosal plexus.[6,7]

The possible mechanisms of inhibition of NANC relaxation by these intravenous anesthetics are considered to be as follows: (1) to inhibit myenteric neurotransmission, thus decreasing or depleting the release of NO (like tetrodotoxin); (2) to inhibit neuronal NO synthase, which distributes in the myenteric plexus; (3) to scavenge NO and prevent it from diffusing into smooth muscle cells; or (4) to accelerate the breakdown of cGMP. Recent evidence has revealed the interactions between anesthetic agents and NO synthase or reactive oxygen species (ROS).[8] In addition, it is well known that superoxide anion inactivates endothelium-derived relaxing factor (EDRF), of which NO is a major form,[9] and thus eliminates the vasodilating ability of EDRF.[10,11] Therefore, we consider modulation of NO synthase activity by anesthetics, or interaction between anesthetics

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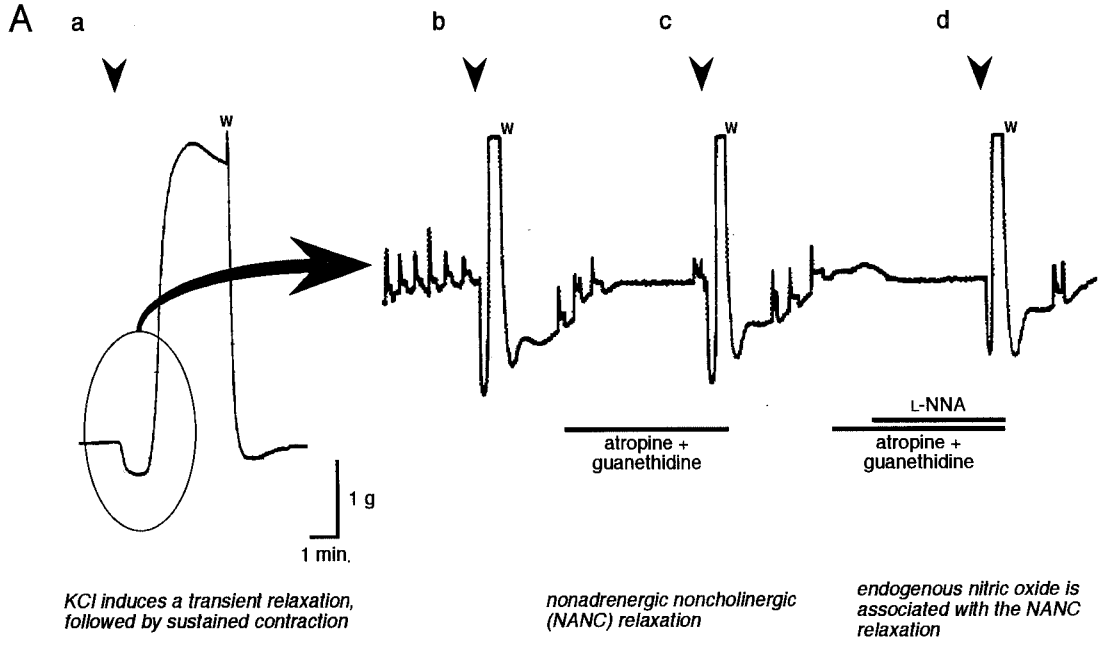
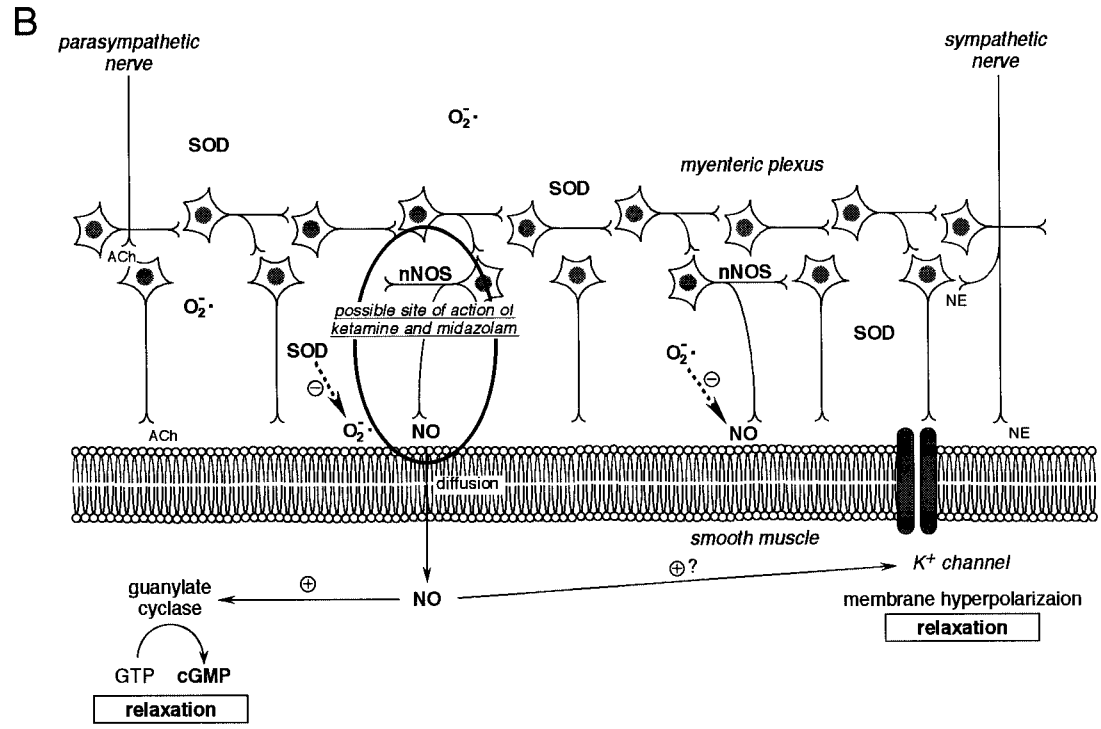
and ROS, especially superoxide anion, to be important, and they are worth further investigation.

Many lines of evidence imply some interactions between anesthetic agents and NO synthase or ROS.[8] Thiopental, ketamine, and midazolam have been reported to inhibit NO synthase activity in the rat brain.[12] On the contrary, the inhibition of NO synthase decreased the anesthetic requirements of thiopental, propofol, and ketamine in *Xenopus laevis*[13] or decreased the minimum alveolar concentration for halothane in rats.[14] Regarding the interactions between ROS and anesthetics, volatile anesthetics have been shown to inhibit endothelium-dependent relaxing factor release[15,16] or to produce ROS[17,18] in vascular smooth muscle.

Therefore, in the current study we investigated the effects of ketamine and midazolam on the relevance of superoxide anion and hydrogen peroxide (H_2O_2) and on NO synthase activity in the inhibition of the NANC relaxation by these anesthetics, using isometric tension recording and the assay of NO synthase activity.





Materials and Methods

Animal Preparation

The Institutional Animal Use Committee approved the experimental protocol. Thirty-three (29 for isometric tension recording, 4 for NO synthase assay) adult male Japanese White rabbits weighing between 2 and 3 kg were anesthetized with intravenous thiopental sodium (50 mg/kg) and killed by exsanguination. The lower part of the esophagus and the stomach were immediately isolated. The esophagogastric junction was opened along the longitudinal axis, and the LES was excised by sharp circular cutting, making strips approximately 2 mm wide and 5 mm long. The mucosa was removed.

Isometric Tension Recording

The method of isometric tension recording of circular muscle was described previously.[5] The strips were vertically fixed between two hooks, and the hook anchoring the upper end was connected to a force–displacement transducer. We adjusted the resting tension to 1.0 g. The strips were suspended in a thermostatically controlled (37.0 ± 0.5°C) 20-ml organ bath containing Krebs-Ringer's solution. The bath fluid was aerated with a mixture of 95% O_2 and 5% CO_2 to keep the pH within 7.35–7.45. Before starting the experiments, the strips were allowed to equilibrate for 60 min in the Krebs solution, which was replaced every 15 min.

Effects of Superoxide Dismutase

First, we examined the effects of superoxide dismutase (SOD), an enzyme that specifically catalyzes the breakdown of superoxide anion to hydrogen peroxide and oxygen,[19–21] and catalase, which catalyzes the breakdown of hydrogen peroxide to H_2O and O_2,[21,22] on ketamine- and midazolam-induced inhibition of the NANC relaxation. The NANC relaxation was induced by 30 mM KCl in the presence of atropine (3×10^{-6} M) and guanethidine (3×10^{-6} M). Atropine and guanethidine were pretreated for at least 10 min. Pretreatment with ketamine (10^{-4} M) or midazolam (10^{-5} M) for 10 min inhibited the NANC relaxation about 30–40%, as described in our previous study.[5] SOD (100, 200, and 400 U/ml) or catalase (100 U/ml) was pretreated for 15 min, and we observed whether the ketamine- and midazolam-induced inhibition of the NANC relaxations could be reversed.

The modification of the NANC relaxation by pretreating with SOD was also examined, using another series of muscle strips.

Effects of NO Donors

Second, we examined the effects of ketamine and midazolam on the relaxations induced by several exogenous NO donors. We used N-ethylethanamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine (diethylamine NONOate; DEA-NO), S-nitrosoglutathione (GSNO), and SNP. The maximal relaxation was obtained by 10^{-4} M papaverine. The concentration of each NO donor producing near-maximal relaxation was used as the highest concentration. We obtained concentration–response relationships of DEA-NO at 3×10^{-7}, 10^{-6}, and 3×10^{-6} M; GSNO at 10^{-5}, 3×10^{-5}, and 10^{-4} M; and SNP at 3×10^{-7}, 10^{-6}, and 3×10^{-6} M. The effects of ketamine (10^{-6}, 10^{-5}, 10^{-4}, and 3×10^{-4} M) and midazolam (10^{-7}, 10^{-6}, 10^{-5}, and 3×10^{-5} M) on the relaxations induced by these NO donors were examined, and concentration–response relationships were obtained. The concentrations of ketamine and midazolam were determined by our previous investigation testing the inhibition of the NANC LES relaxation.[5] Ketamine and midazolam were pretreated for 10 min.

Effects of a Superoxide Generator

The effect of pyrogallol, a superoxide generator, on the NANC and the DEA-NO–induced relaxations was examined. Pyrogallol at concentrations of 10^{-6}, 10^{-5}, 3×10^{-5}, 10^{-4}, and 3×10^{-4} M was pretreated for 10 min, and concentration–response relationships were obtained. The effect of SOD (200 U/ml) on 3×10^{-4} M pyrogallol-induced inhibition of the NANC relaxation was also examined.

Fig. 1. (*A*) Effects of 30 mM KCl, and effects of some antagonists on KCl-induced response on a strip from the lower esophageal sphincter (LES) are shown. KCl induced a transient relaxation followed by sustained contraction induced by membrane depolarization (*a*). In the current study, the component of transient relaxation was magnified to observe (*b*), and that of KCl-induced contraction is overscaled. Pretreating with atropine (3×10^{-6} M), a cholinergic blocking agent, and guanethidine (3×10^{-6} M), an adrenergic blocking agent, the transient relaxation can still be observed, which is called nonadrenergic noncholinergic (NANC) relaxation (*c*). The NANC relaxation is inhibited by pretreating with N^G-nitro-L-arginine (L-NNA; 3×10^{-5} M), a nonspecific inhibitor of nitric oxide synthase, indicating that endogenous nitric oxide is associated with the NANC relaxation (*d*). Arrows indicate the application of KCl. Horizontal bars indicate the presence of atropine and guanethidine or L-NNA; "w" indicates washout. (*B*) Schematic representation of the relation between the myenteric plexus and smooth muscle cells, summarizing the interaction of neurotransmitters mediating the NANC relaxation of the LES is shown. Nitric oxide (NO) mediating the NANC relaxation is released from the myenteric plexus and delivered into smooth muscle cells, which results in the activation of guanylate cyclase and the accumulation of 3',5'-cyclic guanosine monophosphate (cGMP). The NANC relaxation could also be mediated by membrane hyperpolarization, which is induced by the activation of the K^+ channel of smooth muscle. Neuronal NO synthase (nNOS) is considered to localize in the myenteric neurons, nerve fibers, and terminals. Ketamine and midazolam possibly inhibit the process of synthesis, release, or transport of NO, which will be nNOS activation, synaptic transmission, or diffusion within the myenteric plexus. Superoxide anion (O_2^-) reacts with NO and contributes to the instability of NO. Superoxide dismutase (SOD) is an enzyme that specifically catalyzes the breakdown of superoxide anion to hydrogen peroxide and oxygen. ACh = acetylcholine; NE = norepinephrine; GTP = guanosine 5'-triphosphate.

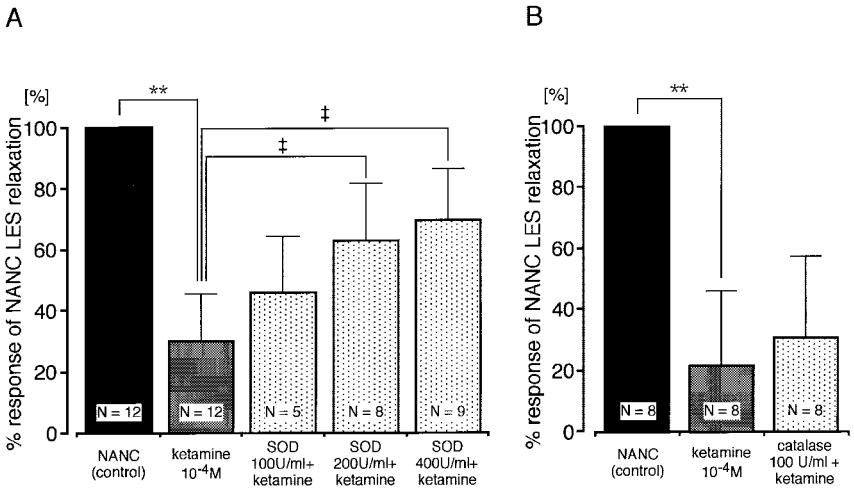


Fig. 2. Effects of increasing concentrations of superoxide dismutase (SOD) (*A*) and 100 U/ml catalase (*B*) on 10^{-4} M ketamine-induced inhibition of 30 mM KCl-induced relaxation in the presence of atropine and guanethidine (nonadrenergic noncholinergic [NANC] relaxation) of strips from the lower esophageal sphincter. Pretreatment with SOD (200, 400 U/ml) significantly reversed the ketamine-induced inhibition of the NANC relaxation, but catalase did not. Each bar represents the mean from tissues from six (*A*) and three (*B*) animals; vertical lines show SD. N indicates the number of strips. **$P <$ 0.01, significantly different from the value in the absence of ketamine. ‡$P < 0.01$, significantly different from the value in the presence of ketamine and in the absence of SOD.

Assay of NO Synthase Activity

For the assay of NO synthase activity, LES strips were quickly excised from another animal, cleaned of connected tissue, and frozen in liquid nitrogen until the assay. A commercial NO synthase quantitative assay kit (Calbiochem®, CN Biosciences, Inc., San Diego, CA) was used. The frozen LES strip was homogenized in 20 vol of homogenization buffer (pH 7.4, 25 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA); the crude homogenates were centrifuged at 4°C for 5 min at 15,000 rpm; and the supernatants were collected. Supernatant samples (10 μl) were added to reaction buffer (40 μl) of the following composition: pH 7.4, 25 mM Tris-HCl buffer, 3 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin mononucleotide, 1 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 1 μCi of L-[^{3}H]arginine. Ketamine at final concentrations of 10^{-5}, 10^{-4}, or 3×10^{-4} M, and midazolam at final concentrations of 10^{-6}, 10^{-5}, or 3×10^{-5} M were added to tubes. The samples were incubated for 30 min at 30°C, and the reaction was discontinued by the addition of ice-cold (2°C) stop buffer (pH 5.5, 50 mM HEPES, 5 mM EDTA). To obtain free L-[^{3}H]citrulline for the determination of enzyme activity, equilibrated resin was added to eliminate excess L-[^{3}H]arginine. The supernatant was assayed for L-[^{3}H]citrulline using a liquid scintillation counter (Wallac 1414 WinSpectral, Turku, Finland). Enzyme activity was expressed as counts per minute per milligram protein. NO synthase activity in the positive control was measured in the presence of 0.6 mM $CaCl_2$ and rat cerebellum extract instead of LES samples. NO synthase activity in the presence of 1 mM N^G-nitro-L-arginine-methylester (L-NAME) served as a negative control.

Drugs

The following drugs were used: potassium chloride from Nacalai Tesque, Kyoto, Japan; and ketamine hydrochloride, atropine sulfate salt, guanethidine monosulfate, papaverine hydrochloride, pyrogallol, DEA-NO, GSNO, SNP dihydrate, SOD from bovine erythrocytes, and catalase from bovine liver from Sigma Chemical, St. Louis, MO. Midazolam was a gift from Yamanouchi Pharmaceutical Co., Tokyo, Japan. Midazolam was dissolved in 1 N HCl and diluted with distilled water to 10 times the initial concentration. The final concentration of HCl in the bath was $< 3 \times 10^{-4}$ N. In a preliminary study, 3×10^{-4} N HCl did not induce any effect on isometric

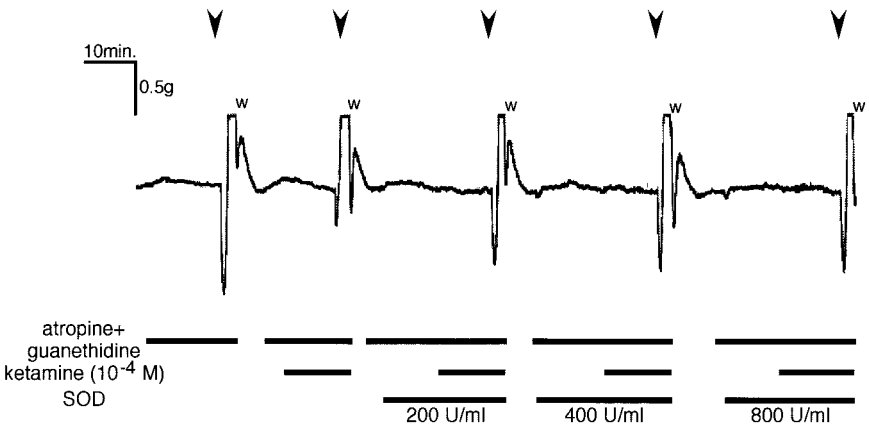


Fig. 3. The effect of increasing concentrations of superoxide dismutase (SOD) on 10^{-4} M ketamine-induced inhibition of the 30 mM KCl-induced relaxation in the presence of atropine and guanethidine (nonadrenergic noncholinergic [NANC] relaxation) of a strip from the lower esophageal sphincter. The ketamine-induced inhibition of the NANC relaxation is reversed in a concentration-dependent manner by pretreating with SOD. To magnify the relaxation, the component of KCl-induced contraction is overscaled. Arrows indicate the application of KCl. Upper horizontal bars indicate the presence of atropine and guanethidine; middle horizontal bars indicate the presence of 10^{-4} M ketamine; lower horizontal bars indicate the presence of SOD; "w" indicates washout.

tension of the muscle. Other drugs were dissolved in distilled water and handled in siliconized glassware.

Statistical Analysis

The results were expressed as mean ± SD. One-way analysis of variance was used to see the differences in the effects of intravenous anesthetics or pyrogallol on the NANC relaxation, effects of SOD on intravenous anesthetics- or pyrogallol-induced inhibition of the NANC relaxation, effects of intravenous anesthetics or pyrogallol on NO donors, and the effects of intravenous anesthetics on NO synthase activity. A Bonferroni test or Scheffé F test (when the number of strips in each group was not equal) was used as a *post hoc* comparison to test for statistical significances between control values and drug-treated ones. For all statistical tests, a P value < 0.05 was regarded as significant.

Results

Ketamine at 10^{-4} M significantly attenuated the NANC relaxation, and pretreatment with SOD (100, 200, and 400 U/ml) for 15 min reversed the ketamine-induced inhibition of the NANC relaxation (fig. 2A). It was significant at 200 and 400 U/ml of SOD ($P < 0.01$), but catalase did not reverse it (fig. 2B). Figure 3 shows a typical tension record displaying a reversal of 10^{-4} M ketamine-induced inhibition of the NANC relaxation with increasing concentrations of SOD. The maximal reversal of the NANC relaxation by SOD was observed at 400 U/ml. Ketamine inhibited the relaxation induced by DEA-NO (fig. 4A) and GSNO (fig. 4B), and the inhibition was significant at 3×10^{-4} M ketamine for all of the concentrations of DEA-NO and GSNO ($P < 0.05$; $P < 0.01$ at 3×10^{-4} M ketamine for 3×10^{-6} M DEA-NO and 10^{-4} M GSNO), and at 10^{-4} M ketamine for 3×10^{-6} M DEA-NO– and 10^{-4} M GSNO-induced relaxation ($P < 0.05$). Ketamine did not, however, affect the relaxation induced by SNP (fig. 4C).

The NANC relaxation itself was not affected by pretreating with increasing concentrations of SOD (100, 200, and 400 U/ml; table 1).

The midazolam-induced inhibition of the NANC relaxation was reversed neither by SOD (fig. 5A) nor by catalase (fig. 5B). Midazolam did not affect the relaxations induced by DEA-NO (fig. 6A), GSNO (fig. 6B), or SNP (fig. 6C), except for a significant inhibition at 3×10^{-5} M midazolam for 3×10^{-6} M DEA-NO–induced relaxation ($P < 0.01$).

The NO synthase activity was concentration-dependently suppressed by midazolam at 10^{-5} M ($P < 0.05$) and 3×10^{-5} M ($P < 0.01$; fig. 7A), and midazolam at 3×10^{-5} M suppressed the NO synthase activity almost to the negative control value obtained by L-NAME, a nonspecific inhibitor of NO synthase. There was no marked effect of ketamine on NO synthase activity (fig. 7B).

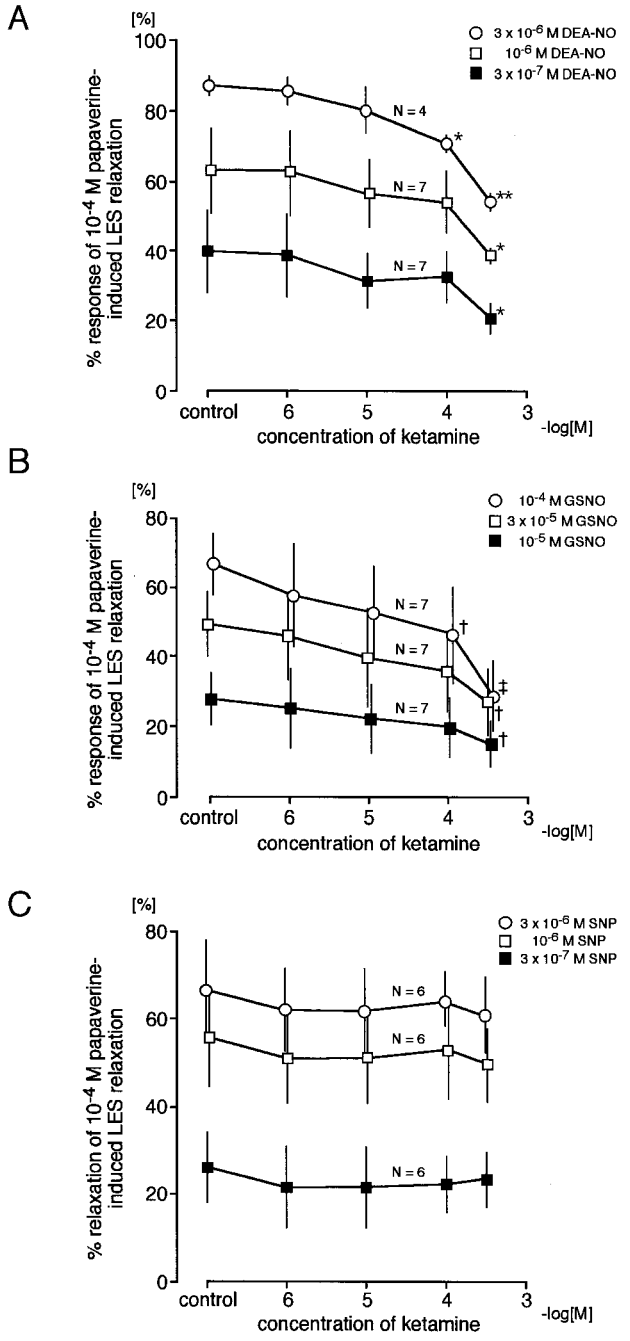


Fig. 4. The effect of ketamine on diethylamine NONOate (DEA-NO)– (*A*), S-nitrosoglutathione (GSNO)- (*B*), and sodium nitroprusside (SNP)- (*C*) induced relaxations of strips from the lower esophageal sphincter. Ketamine concentration-dependently inhibited the DEA-NO– and GSNO-induced relaxations but not the SNP-induced relaxation. The curve is expressed as a % response of 10^{-4} M papaverine-induced relaxation in the absence of ketamine. Each point represents the mean from tissues from two animals (*A–C*); vertical lines show SD. N indicates the number of strips. *$P < 0.05$, **$P < 0.01$, significantly different from each of the values induced by DEA-NO in the absence of ketamine. †$P < 0.05$, ‡$P < 0.01$, significantly different from each of the values induced by GSNO in the absence of ketamine.

Table 1. Effects of Increasing Concentrations of Superoxide Dismutase on NANC Relaxation of Isolated Rabbit Lower Esophageal Sphincter Strips

NANC Relaxation (control)	Superoxide Dismutase		
	100 U/ml	200 U/ml	400 U/ml
100 (N = 7)	99.6 ± 10.5 (N = 7)	88.8 ± 17.7 (N = 6)	85.3 ± 5.8 (N = 6)

Values are expressed as percent response of nonadrenergic noncholinergic (NANC) relaxation. Each value represents the mean ± SD from tissues from four animals. N indicates the number of strips. The NANC relaxation was not affected by pretreating with superoxide dismutase.

The application of pyrogallol, a superoxide generator, on the NANC and DEA-NO–induced relaxation mimicked the action of ketamine. Pyrogallol at high concentrations inhibited the NANC relaxation (fig. 8A), and the inhibition was significant at 10^{-4} M ($P < 0.01$) and 3×10^{-4} M ($P < 0.01$). Figure 8B shows the significant reversal of the 3×10^{-4} M pyrogallol-induced inhibition of the NANC relaxation by pretreating with 200 U/ml SOD ($P < 0.01$). Pyrogallol at high concentrations also inhibited the DEA-NO–induced relaxation (fig. 9), and the inhibition was significant at 10^{-4} M ($P < 0.05$) and 3×10^{-4} M ($P < 0.01$) for 3×10^{-6} M DEA-NO, and at 3×10^{-4} M for 10^{-6} M ($P < 0.01$) and 3×10^{-7} M ($P < 0.05$) DEA-NO.

Discussion

Superoxide anion has been demonstrated to react with NO and contribute to the instability of NO,[10,11] thus inhibiting endothelium-dependent vascular smooth muscle relaxation.[23,24] In the LES, the antioxidant enzyme system appears to play a role in the maintenance of LES function.[25] In the current study, the ketamine-induced inhibition of the NANC relaxation was partly reversed by pretreating with SOD but was not reversed by catalase. The NANC relaxation itself was not affected by pretreating with SOD. As SOD is known to be unable to penetrate into cells because of its higher molecular weight,[26] it is suggested that ketamine may possibly produce superoxide anion extracellularly and thus inactivate endogenous NO mediating the NANC relaxation. The relaxations induced by two exogenous NO donors, DEA-NO and GSNO, were significantly inhibited by pretreating with ketamine. In addition, a superoxide generator pyrogallol mimicked the action of ketamine on the NANC and DEA-NO–induced relaxations; pyrogallol significantly inhibited both of the relaxations, and the pyrogallol-induced inhibition of the NANC relaxation was reversed by pretreating with SOD. These findings support the hypothesis that ketamine inhibits NANC LES relaxation, at least in part, *via* the extracellular generation of superoxide anion and not *via* the hydrogen peroxide pathway. Ketamine did not affect the SNP-induced relaxation in the current study. The reason for this result may be that extracellular superoxide anion could not inactivate intracellular NO-mediated relaxation, as SNP has been reported to be metabolized into NO inside the cells.[27] Ketamine did not alter the activity of NO synthase in the current study.

The reversal of the ketamine-induced inhibition of the NANC relaxation by SOD, however, was limited to about 60–70% of the NANC relaxation, and we previously observed that the decrease in cGMP content by ketamine was less than that induced by midazolam.[5] Therefore, the superoxide generation by ketamine would be one of the mechanisms mediating the inhibition of the NANC relaxation. Ketamine has been reported to inhibit the K^+ channel of the peripheral nerve membrane.[28] Immunoreactivity of *N*-methyl-D-aspartate (NMDA) receptor was observed in the submucosal and myenteric plexus of the guinea pig gut[29] and in the rat esophageal plexus neuron,[30] and pharmacologic methods also revealed a subtype of NMDA receptor in the guinea pig ileum.[31] According to these lines of evidence, we cannot exclude the possibility that ketamine inhibits smooth

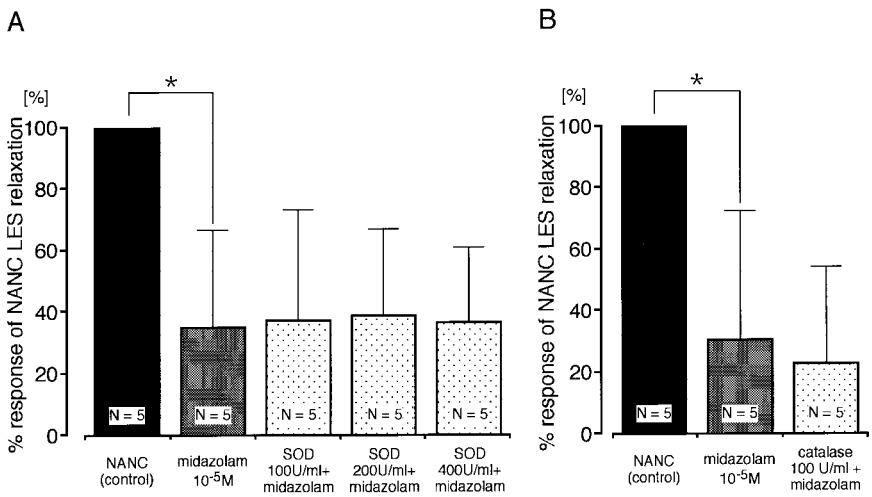


Fig. 5. Effects of increasing concentrations of SOD (*A*) and 100 U/ml catalase (*B*) on 10^{-5} M midazolam-induced inhibition of 30 mM KCl-induced relaxation in the presence of atropine and guanethidine (nonadrenergic noncholinergic [NANC] relaxation) of strips from the lower esophageal sphincter. Pretreatment with SOD (100, 200, and 400 U/ml) or catalase did not reverse the midazolam-induced inhibition of the NANC relaxation. Each bar represents the mean from tissues from two animals (*A, B*); vertical lines show SD. N indicates the number of strips. *$P < 0.05$, significantly different from the value in the absence of midazolam.

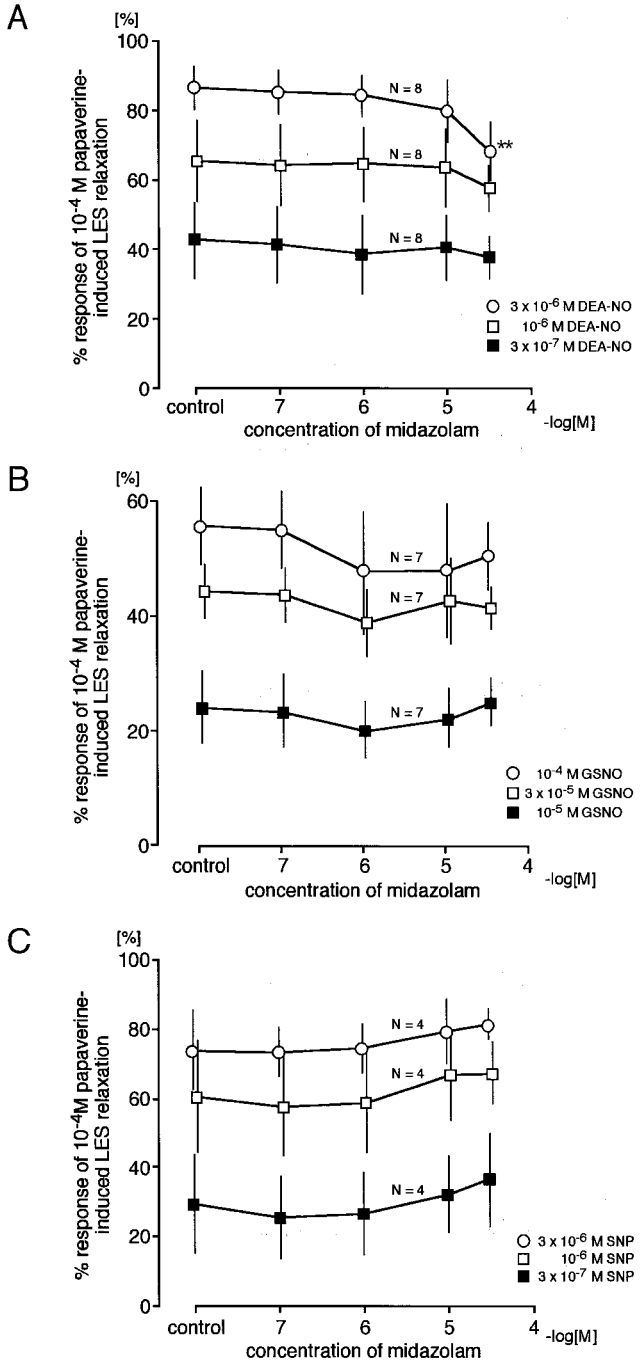


Fig. 6. The effect of midazolam on diethylamine NONOate (DEA-NO)– (*A*), S-nitrosoglutathione (GSNO)- (*B*), and sodium nitroprusside (SNP)- (*C*) induced relaxations of strips from the lower esophageal sphincter. Midazolam did not affect the relaxations induced by these NO donors, except for the inhibition of 3×10^{-6} M DEA-NO–induced relaxation at 3×10^{-5} M. The curve is expressed as a % response of 10^{-4} M papaverine-induced relaxation in the absence of midazolam. Each point represents the mean from tissues from three (*A, B*) and two (*C*) animals; vertical lines show SD. N indicates the number of strips. **$P < 0.01$, significantly different from the value induced by 3×10^{-6} M DEA-NO in the absence of midazolam.

muscle K^+ channels or inhibits NMDA receptors of the myenteric neurons. In tracheal smooth muscle, ketamine has been reported to block the NMDA receptor of smooth muscle, but the relaxing effect is independent of the NMDA receptor.[32]

Few articles have referred to interactions between ketamine and NO in smooth muscle, despite several investigations having revealed interactions between ketamine and the voltage-dependent Ca^{2+} channel[33–37] or intracellular Ca^{2+} stores.[37,38] Recent evidence revealed that ketamine inhibits NO formation in the rat aorta[39] and the NO-mediated vasorelaxant component in the canine pulmonary artery.[40] The results of the former investigation by Miyawaki *et al.*[39] showed that ketamine inhibited endothelium-dependent relaxation induced by acetylcholine and acetylcholine-stimulated cGMP levels, but it did not inhibit SNP-induced relaxation in the rat aorta; these observations may be explained by superoxide generation by ketamine. Other investigators, however, have recently reported conflicting results indicating an antioxidant property of ketamine that the anesthetics inhibited NADPH-stimulated lipid peroxidation and hydrogen peroxide production using cytochrome P450 from rat liver *in vitro*.[41] They also proposed a relationship between this antioxidant capacity and the affinity of the NMDA receptor binding site of several NMDA antagonists.[41] Although the experimental object, method, and condition differ from our study, further experiments will be needed to clarify the interaction between ROS and ketamine.

Midazolam did not alter the relaxations induced by NO donors (except 3×10^{-5} M midazolam for 10^{-5} M GSNO-induced relaxation), and midazolam-induced inhibition of the NANC relaxation was not reversed by SOD or catalase. Thus, superoxide anion and hydrogen peroxide are not associated with the midazolam-induced inhibition of the NANC relaxation. Midazolam significantly inhibited the activity of NO synthase extracted from the LES, and the inhibition at 3×10^{-5} M was nearly equal to the negative control value induced by L-NAME, a nonspecific inhibitor of NO synthase. Midazolam thus inhibits NANC LES relaxation by suppressing the activity of NO synthase. Midazolam has been reported, however, to have no effect on endothelium-dependent relaxation and SNP-induced relaxation or on acetylcholine-stimulated cGMP level,[39] and to directly relax tracheal smooth muscle contracted by acetylcholine.[42] To our knowledge, our study is the first observation that midazolam inhibits the NO synthase activity of smooth muscle. A previous clinical investigation showing that midazolam induced abnormal esophageal motility as strong esophageal contractions in healthy adult volunteers[43] may be explained by the suppression of NO synthase of esophageal smooth muscle.

It has been demonstrated that neuronal NO synthase mediates NANC LES relaxation in mice,[7] and immuno-

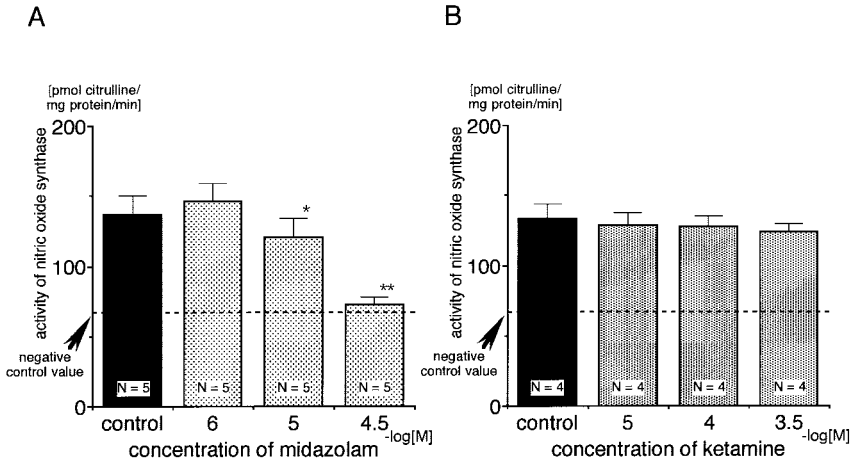


Fig. 7. Effects of increasing concentrations of midazolam (*A*) and ketamine (*B*) on the activity of nitric oxide (NO) synthase, which extracted from strips of the lower esophageal sphincter. Midazolam concentration-dependently inhibited the NO synthase activity, whereas ketamine did not affect it. Midazolam at 3×10^{-5} M inhibited the NO synthase activity almost identically to that induced by L-NMMA (negative control). Each column represents the mean from tissues from three (*A, B*) animals; vertical lines show SD. N indicates the number of strips. *$P < 0.01$, **$P < 0.05$ significantly different from the value in the absence of midazolam.

histochemical studies have revealed that NO synthase immunoreactivity is evident at the myenteric plexus in the rat intestine[44] and at the myenteric plexus and the motor nerve terminals in the mouse esophagus.[45] Therefore, we consider the NO synthase activity in the current study as mainly derived from the myenteric neurons. However, we could not differentiate the NO synthase activity in the myenteric neurons from that in the smooth muscle cells.

Substantial evidence exists indicating that the L-arginine–NO pathway generates the NANC neurotransmitter, which mediates relaxations of smooth muscle in the respiratory, gastrointestinal, and urogenital tracts, as the NANC relaxation could be suppressed by NO synthase inhibition.[46] The nature of nitrergic neurotransmission in smooth muscle has been well investigated in the anococcygeus, retractor penis, and gastric fundus. Many investigations have revealed that superoxide generators and direct NO scavengers can inhibit relaxations to exogenous NO,[47,48] but they have little effect on relaxations to nitrergic stimulation.[48,49] Some hypotheses have been formulated to explain this paradox, and recently the importance of a tissue enzyme, Cu/Zn SOD, has been emphasized.[50,51] NANC neurotransmitter is protected by high levels of Cu/Zn SOD, and the inhibition of this enzyme by diethyldithiocarbamate (DETCA) increases the susceptibility to destruction by superoxide anions.[52] In the current study, a superoxide generator, pyrogallol, inhibited the NANC relaxation and the relaxations induced by NO donors. However, high concentrations of pyrogallol were needed to inhibit these relaxations. This finding is consistent with other studies using such high concentrations of superoxide generators to inhibit NANC relaxation in the DETCA-untreated anococcygeus.[49,50,53] Although the role of Cu/Zn SOD in LES is not fully elucidated, Cu/Zn SOD appears to play a role in modulating the normal esophageal motor function.[54] Ketamine may have an ability to inhibit the activity of this enzyme and thus inhibit the NANC LES relaxation. Further investigation will be needed to clarify the role of Cu/Zn SOD in LES and the effect of anesthetics on Cu/Zn SOD.

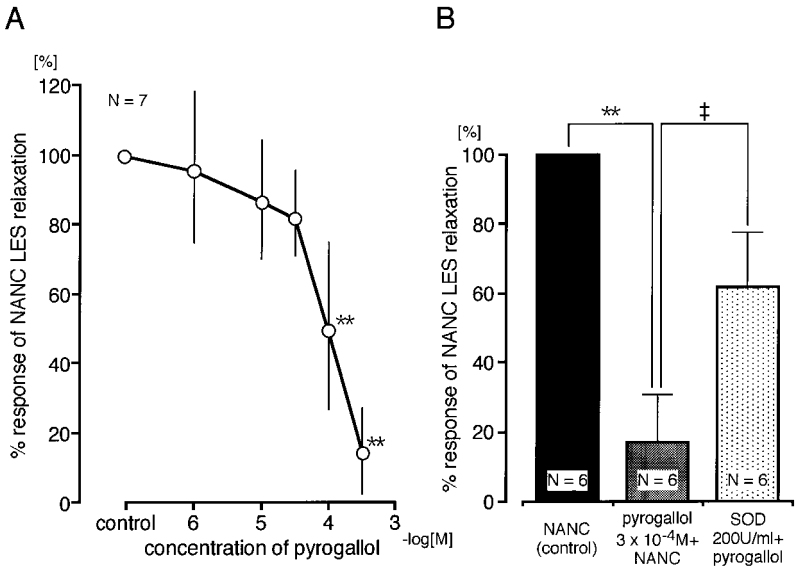


Fig. 8. The concentration–response relationship of pyrogallol on 30 mM KCl-induced relaxation in the presence of atropine and guanethidine (nonadrenergic noncholinergic [NANC] relaxation) (*A*), and the effect of superoxide dismutase (SOD; 200 U/ml) on 3×10^{-4} M pyrogallol-induced inhibition of the NANC relaxation (*B*) of strips from the lower esophageal sphincter. Pyrogallol concentration-dependently inhibited the NANC relaxation, and 3×10^{-4} M pyrogallol-induced inhibition of the NANC relaxation was significantly reversed by pretreating with SOD. Each point or bar represents the mean from tissues from five (*A*) and three (*B*) animals; vertical lines show SD. N indicates the number of strips. **$P < 0.01$, significantly different from the value in the absence of pyrogallol. ‡$P < 0.01$, significantly different from the value in the presence of pyrogallol and in the absence of SOD.

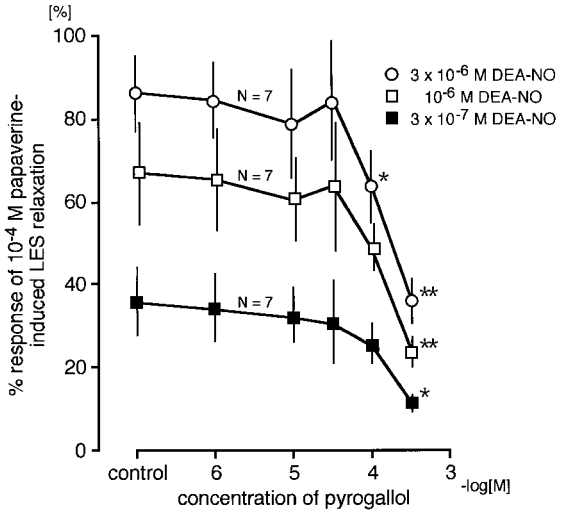


Fig. 9. The concentration–response relationship of pyrogallol on diethylamine NONOate (DEA-NO)–induced relaxations of strips from the lower esophageal sphincter. Pyrogallol concentration-dependently inhibited the DEA-NO–induced relaxation. The curve is expressed as a % response of 10^{-4} M papaverine-induced relaxation in the absence of pyrogallol. Each point represents the mean from tissues from five animals; vertical lines show SD. N indicates the number of strips. *$P < 0.05$, **$P < 0.01$, significantly different from each of the values induced by DEA-NO in the absence of pyrogallol.

The observations of our present *in vitro* study should not be transferred to clinical anesthetic practice as it is because depolarization using KCl is not a natural stimulus for the NANC relaxation. The endogenous NO, however, is certainly involved in the KCl-induced NANC relaxation in rabbit LES, and in humans, NO is considered to be associated with the swallowing-induced peristaltic response of the esophageal body and LES.[55] Therefore, ketamine and midazolam may possibly interfere this physiologic response of the LES by different mechanisms and may increase the risk of regurgitation.

In conclusion, it is suggested that ketamine inhibits NANC LES relaxation, at least in part, by the extracellular production of superoxide anion, and that midazolam inhibits it by the inhibition of NO synthase activity.

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