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Endothelium-independent and -dependent vasoactivity of 6-nitronorepinephrine

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Abstract

Vasoactivities of 6-nitronorepinephrine were investigated using rat aorta. 6-Nitronorepinephrine (> 100 μM) caused dose-dependent contraction in both endothelium-intact and -denuded aorta, although the latter showed greater contraction than the former. Prazosin (> 3 nM), an α_1-adrenoceptor antagonist, attenuated significantly the 6-nitronorepinephrine-induced contractions, thereby suggesting the α_1-adrenoceptor involvement. Aortic rings prepared from reserpine-pretreated rats showed the 6-nitronorepinephrine-induced a contraction to the extent similar to those from untreated rats, suggesting that endogenous norepinephrine does not play a role in the 6-nitronorepinephrine-induced contraction. 6-Nitronorepinephrine (> 10 μM) potentiated norepinephrine-induced contraction only in the presence of endothelium. The augmentation was attenuated by catalase (1200 U/ml). H_2O_2 (10–300 μM) augmented the norepinephrine-induced contraction only in the endothelium-intact rat aortic rings. 6-Nitronorepinephrine attenuated significantly acetylcholine-induced relaxation. Catalase prevented the 6-nitronorepinephrine-induced inhibition of the acetylcholine-induced relaxation. These results suggest that 6-nitronorepinephrine has a weak α_1-adrenoceptor agonistic property and that the endothelium-dependent potentiation by 6-nitronorepinephrine of the norepinephrine-induced contraction is mediated through production of H_2O_2.

Keywords: Nitric oxide (NO); Catecholamine; Blood vessel; Catalase; Hydrogen peroxide

1. Introduction

Nitric oxide (NO) is a ubiquitous molecule which plays important roles in human physiology and pathology (Beckman, 1996). Interactions between NO and molecular constituents such as heme iron and sulfhydryl group modulate protein functions (Ignarro, 1991; Stamler, 1994). Much less is known about NO-related modulation of neurotransmitter molecules.

Exposure of catecholamines to NO in aerated phosphate buffer at room temperature leads to 6-nitration of catecholamines in vitro (De la Bretèche et al., 1994; D'Ischia and Constantini, 1995). We have identified 6-nitronorepinephrine in vivo (Shintani et al., 1996). 6-Nitronorepinephrine inhibits norepinephrine reuptake into synaptosomes and catechol-*O*-methyltransferase activities (Shintani et al., 1996). However, biological activities of 6-nitronorepinephrine have not been fully addressed in isolated live tissues, thereby obtaining insights into cellular mechanisms of 6-nitronorepinephrine action. Herein, we describe the vasoactivities of 6-nitronorepinephrine by the use of rat aortic rings.

2. Materials and method

2.1. Materials

Racemic 6-nitronorepinephrine was synthesized in Santen Pharmaceutical (Osaka, Japan). The purity in terms of the racemic 6-nitronorepinephrine was greater than 95%. Acetylcholine, norepinephrine, catalase, and reserpine were purchased from Sigma Chemical (St. Louis, MO, USA) Reserpine was dissolved in a minimum volume of warm glacial acetic acid and diluted with saline. Prazosin was donated by Pfizer, Tokyo, Japan. Analytical grade of hydrogen peroxide (H_2O_2) was purchased from Kanto Chemical (Tokyo, Japan).

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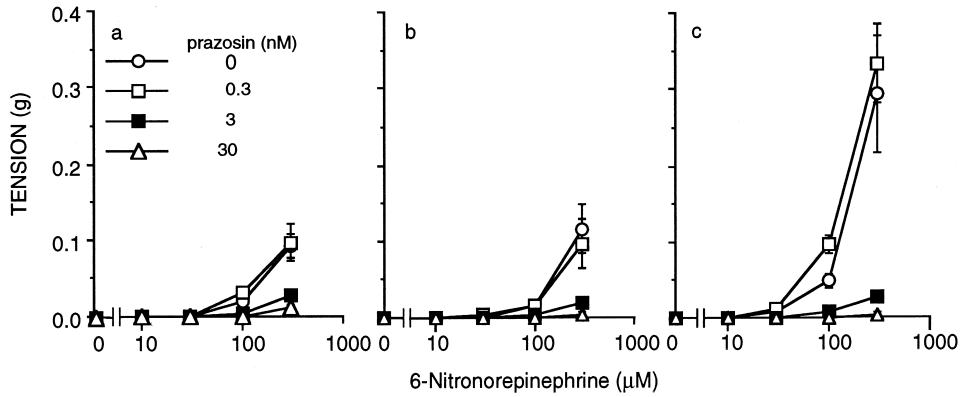

Fig. 1. Contractile effect of 6-nitronorepinephrine on rat aorta and the inhibition by prazosin. The dose–response curves were obtained by stepwise additions of 6-nitronorepinephrine. Each concentration of prazosin was added 30 min before the 6-nitronorepinephrine addition. Points and bars are the means ± S.E.M. of data from four aortic rings. a, endothelium-intact rings, prepared from control rats; b, endothelium-intact rings, prepared from reserpine-pretreated rats; c, endothelium-denuded rings, prepared from control rats. Prazosin at concentration higher than 3 nM significantly ($P < 0.05$) inhibited the 6-nitronorepinephrine-induced contraction.

2.2. Measurement of tension in rat aortic rings

Male Wistar rats (250–300 g) were killed by decapitation. Rings of thoracic aorta were prepared from rats as described previously (Nakaki et al., 1985). A ring was mounted at 37°C in an organ chamber (10 ml) containing a Krebs–Ringer bicarbonate buffer of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 25; and glucose 11. A mixture of 95% O_2/5% CO_2 was bubbled continuously through the buffer. Tensions were monitored with an isometric transducer (Nihonkoden, Tokyo, Japan) and recorded on an ink-writing oscillograph (Nihonkoden). The initial resting force applied to the aortic ring was 1.0 g. The aortic rings were allowed to reach a stable tension before exposure to the drugs. When necessary, the organ chambers were washed at least three times at 5-min intervals. The presence of an intact endothelium was confirmed by the observation that acetylcholine (10 μM) caused full relaxation of the aortic rings. When a dose–response curve was constructed, drug concentration was increased 3-fold at each step, with each addition being made only after the contraction elicited by the previous addition had reached a steady state. The maximal, tested concentration of 6-nitronorepinephrine was 300 μM because of a limited supply of the drug.

In other experiments, the endothelium was removed by inserting a small forceps into the lumen and by gently rolling the ring back and forth several times (Schini and Vanhoutte, 1991). Aortic rings which showed the acetylcholine-induced relaxation after this procedure were considered to have residual endothelium and were not used for the endothelium-denuded ring experiments.

For reserpine-pretreated rats, the drug (5 mg/kg, s.c.) was injected twice, 48 and 24 h, before killing. This treatment depletes approximately 90% of norepinephrine in blood vessels (Okada et al., 1993).

2.3. Statistics

Results are presented as means ± S.E.M. Statistical significance was evaluated by analysis of variance and the *t*-test.

3. Results

3.1. 6-Nitronorepinephrine-induced contraction

6-Nitronorepinephrine induced a dose-dependent contraction in endothelium-intact rings (Fig. 1a). Prazosin, a

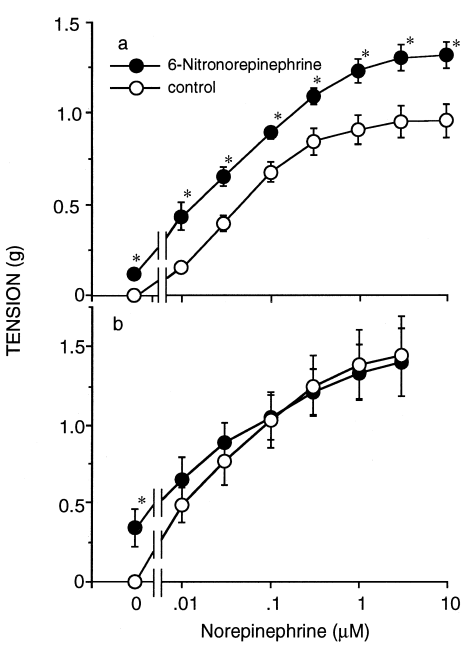

Fig. 2. Effect of 6-nitronorepinephrine on norepinephrine-induced contraction of rat aorta. The dose–response curves were obtained by stepwise additions of norepinephrine. 6-Nitronorepinephrine (300 μM) was added 5 min before the addition of norepinephrine. Points and bars are the means ± S.E.M. of data from four aortic rings. a, with intact endothelium; b, without endothelium. * $P < 0.05$ vs. control.

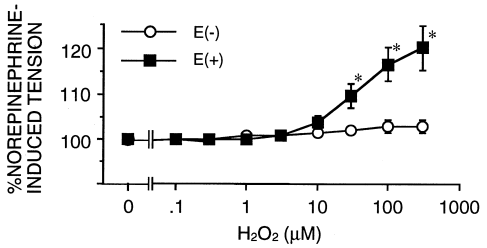

Fig. 3. Effect of H_2O_2 on norepinephrine-induced contraction of rat aorta. The aortic rings were contracted by 3 μM norepinephrine and the tension was allowed to reach a constant level. Then, dose–response curves were obtained by stepwise cumulative addition of H_2O_2. E(+), endothelium-intact rings; E(−), endothelium-denuded rings. One hundred percent tensions denote those just before the addition of H_2O_2 and were: E(−) control, 1.14±0.16; E(+) control, 0.79±0.11 g. Points and bars are the means±S.E.M. of data from four rings. $* P < 0.05$ vs. the corresponding E(−).

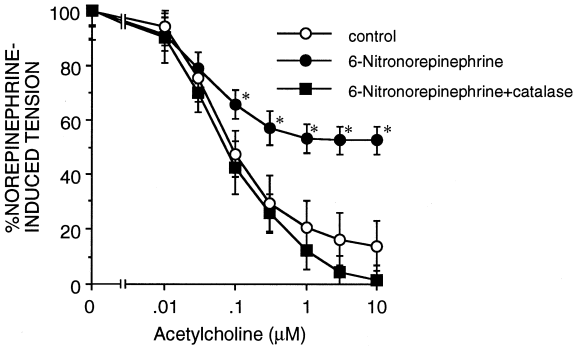

Fig. 5. Attenuation by 6-nitronorepinephrine of acetylcholine-induced relaxation of norepinephrine-precontracted rat aorta and its reversal by catalase. The endothelium-intact aortic rings were contracted by 3 μM norepinephrine and the tension was allowed to reach a constant level. 6-Nitronorepinephrine (300 μM) and catalase were added 5 min and 30 min before the addition of norepinephrine, respectively. The dose–response curves of acetylcholine were made by stepwise cumulative addition of acetylcholine. Points and bars are the means±S.E.M. of data from four rings. $* P < 0.05$ vs. control and 6-nitronorepinephrine+catalase.

nonspecific α_1-adrenoceptor antagonist, markedly inhibited the 6-nitronorepinephrine-induced contraction at concentrations higher than 3 nM. The pretreatment with reserpine did not affect the 6-nitronorepinephrine-induced contraction (Fig. 1b). Removal of endothelium enhanced the 6-nitronorepinephrine response (Fig. 1c).

3.2. Endothelium-dependent potentiation by 6-nitronorepinephrine of norepinephrine-induced precontraction

6-Nitronorepinephrine at the concentration of 300 μM not only shifted the dose–response curve of norepinephrine to the left but enhanced the maximum contraction of norepinephrine (Fig. 2a). On the contrary, no enhancement of the norepinephrine-induced contraction was observed in the endothelium-denuded rings (Fig. 2b).

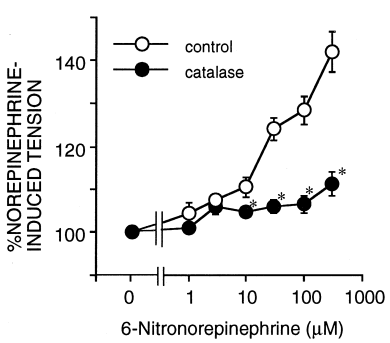

Fig. 4. Effect of catalase on 6-nitronorepinephrine-induced potentiation of norepinephrine-induced-contraction of endothelium-intact rat aorta. Catalase (1200 U/ml) was added 30 min before norepinephrine. The aortic rings were contracted by 3 μM norepinephrine and the tension was allowed to reach a constant level. Then, dose–response curves were obtained by stepwise cumulative addition of 6-nitronorepinephrine. One hundred percent tensions denote those just before the addition of 6-nitronorepinephrine and were: control, 0.61±0.07; catalase, 0.72±0.07 g. Points and bars are the means±S.E.M. of data from four rings. $* P < 0.05$ vs. control.

3.3. Involvement of H_2O_2 in 6-nitronorepinephrine-induced potentiation

In rat aorta, we found that H_2O_2 potentiated the norepinephrine-induced contraction of the endothelium-intact but not endothelium-denuded rings (Fig. 3). Fig. 4 shows the dose–response curve of 6-nitronorepinephrine, which enhanced the norepinephrine-induced contraction. Catalase, which degrades H_2O_2, significantly inhibited the 6-nitronorepinephrine-induced augmentation of norepinephrine-induced contraction (Fig. 4).

3.4. Attenuation by 6-nitronorepinephrine of acetylcholine-induced relaxation

Without 6-nitronorepinephrine, acetylcholine induced a dose-dependent relaxation of norepinephrine-induced contraction, restoring the basal tone. In the presence of 6-nitronorepinephrine, acetylcholine induced relaxation but not to the basal level (Fig. 5). The presence of catalase completely reversed the 6-nitronorepinephrine-blunted relaxation.

4. Discussion

6-Nitronorepinephrine induced a dose-dependent contraction in the endothelium-intact rings. Prazosin, an α_1-adrenoceptor antagonist, markedly inhibited the 6-nitronorepinephrine-induced contraction at concentrations (3 and 30 nM) which are known to block specifically α_1-adrenoceptors. This suggests that α_1-adrenoceptors are

involved in the action of 6-nitronorepinephrine and that nitration at the 6th position of norepinephrine largely loses the potency (at least 300 fold less) as an α_1-adrenoceptor agonist.

Since 6-nitronorepinephrine inhibits the activities of catechol-*O*-methyltransferase ($IC_{50} = 7.5$ μM) and norepinephrine uptake to rat synaptosomes ($IC_{50} = 31$ μM)(Shintani et al., 1996), it is possible that α_1-adrenoceptors are stimulated by endogenous norepinephrine which remains in the adventitia in aortic rings (Karaki and Urakawa, 1977; Sudhir and Angus, 1990). To exclude this possibility, we tested the effects of reserpine, which depletes endogenous catecholamines in blood vessels (Okada et al., 1993). The pretreatment with reserpine, which depletes approximately 90% of norepinephrine in blood vessels (Okada et al., 1993), did not affect the 6-nitronorepinephrine-induced contraction, thereby ruling out the role of endogenous norepinephrine. It is concluded that 6-nitronorepinephrine has a property of α_1-adrenoceptor agonist with potencies at least 300 fold less than those of norepinephrine.

Removal of endothelium enhanced the 6-nitronorepinephrine response. This shows that the contraction being induced by 6-nitronorepinephrine alone is endothelium-independent. It is consistent with the previous observation that the removal of the endothelial cells enhanced the reactivity of rat aorta to α-adrenoceptor agonists (Lues and Schümann, 1984; Nakaki et al., 1992).

Without endothelium, 6-nitronorepinephrine did not affect the maximal contraction of the norepinephrine-induced contraction. Since both 6-nitronorepinephrine and norepinephrine activate α_1-adrenoceptors to contract the smooth muscles, one would not expect that the maximal effects should be increased by simultaneous addition of the two drugs.

In the presence of endothelium, however, 6-nitronorepinephrine not only shifted the dose–response curve of norepinephrine to the left but enhanced the maximal effect of norepinephrine. Therefore, endothelium plays a role in the augmentation. The endothelium dependency, however, does not necessarily mean direct interaction between 6-nitronorepinephrine and endothelium.

6-Nitronorepinephrine inhibits the activities of catechol-*O*-methyltransferase and norepinephrine uptake to rat synaptosomes (Shintani et al., 1996). The non-neuronal uptake1 sites, which are associated with intracellular catechol-*O*-methyltransferase and/or monoamine oxidase, are present in vascular endothelium (Trendelenburg, 1990). Therefore, one possible interpretation is that 6-nitronorepinephrine-elicited augmentation of norepinephrine-induced contraction is due to inhibitory activities of uptake1 sites. However, since the inhibition of uptake1 sites eventually leads to the increased concentration of norepinephrine, the augmentation of the maximal effect of norepinephrine might be difficult to be explained by the sole inhibition of uptake1 sites.

Although H_2O_2 relaxes blood vessels (Burke and Wolin, 1987; Zembowicz et al., 1993) under some experimental conditions, it was shown that H_2O_2 at less than 1 mM concentrations causes an endothelium-dependent contraction (Katusic et al., 1993). H_2O_2 concentrations in vivo depend on effectiveness of H_2O_2-removal systems: less than 0.1 μM in rat liver and 10–25 μM in the lens of human eyes (Halliwell and Gutteridge, 1989). High concentrations more than 1 mM might have no relevance to physiological or to pathophysiological conditions in vivo. In rat aorta, we found that H_2O_2 (10–300 μM)-potentiated the norepinephrine-induced contraction of the endothelium-intact but not -denuded rings (Fig. 3). Therefore, H_2O_2 is an endothelium-dependent potentiator of rat aortic contraction. We examined whether 6-nitronorepinephrine-induced potentiation involves H_2O_2. Catalase, an H_2O_2-degrading enzyme, which does not affect norepinephrine-induced contraction itself (Sunman et al., 1993), inhibited significantly the 6-nitronorepinephrine-induced augmentation of the norepinephrine-induced contraction (Fig. 4). Monoamine oxidase B, which is present in aortic endothelial cells (Meresse et al., 1989), produces H_2O_2 as a byproduct. It is unlikely, however, that the monoamine oxidase B is the source of 6-nitronorepinephrine-induced generation of H_2O_2, since pargyline, an inactivator of monoamine oxidase (Fowler et al., 1982), did not abolish the potentiating effect of 6-nitronorepinephrine (data not shown).

Catalase did not affect the α_1-adrenoceptor-mediated contraction, which is induced by 6-nitronorepinephrine alone in the presence of endothelium and without norepinephrine (data not shown), suggesting that α_1-adrenoceptor-mediated contraction does not involve H_2O_2 generation. A question may arise why 6-nitronorepinephrine alone might not trigger H_2O_2 generation without norepinephrine. Aerobic glycolysis is associated with membrane energy-dependent functions and oxidative metabolism correlates with vascular contractility (Zhang and Paul, 1994). One of mechanisms underlying the effect, therefore, may be related to increases in mitochondrial respiration and superoxide generation by norepinephrine. The norepinephrine-induced contraction possibly triggers an amplified formation of quinone/semiquinone and H_2O_2 from 6-nitronorepinephrine. These mechanisms are a reminiscence of a neurotoxin, 6-hydroxydopamine, which is the hydroxylated derivative at the 6th position of dopamine and produces quinone/semiquinone and H_2O_2 (Heikkila and Cohen, 1971).

Without 6-nitronorepinephrine, acetylcholine induced a dose-dependent relaxation of the norepinephrine-induced contraction, restoring the basal tone. In the presence of 6-nitronorepinephrine, the acetylcholine-induced relaxation was attenuated significantly (Fig. 5). Since acetylcholine-induced relaxation is due to endothelium-derived relaxation factor (EDRF)/NO, this may imply that 6-nitronorepinephrine inactivates NO synthase or EDRF/NO

in the presence of endothelium and norepinephrine. Catalase has been known not to affect the EDRF-mediated relaxation (Silin et al., 1985; Sunman et al., 1993). The presence of catalase reversed completely the 6-nitronorepinephrine-blunted relaxation, suggesting, therefore, that H_2O_2 is involved in the attenuation by 6-nitronorepinephrine of the acetylcholine-induced relaxation. This is consistent with the reports that NO and H_2O_2 act antagonistically (Langenstroer and Pieper, 1992; Chang et al., 1996; Mcquaid et al., 1996). This mechanism also may account, at least in part, for the augmentation by 6-nitronorepinephrine of the norepinephrine-induced contraction; H_2O_2 counteracts NO, which is released from endothelium in the presence of norepinephrine, thereby norepinephrine-induced contraction being enhanced.

Since 6-nitronorepinephrine is probably generated in discrete brain areas, in which adrenergic and nitrergic neurons are juxtaposed, the vasoactivities of 6-nitronorepinephrine are probably of little physiological significance in vessels in vivo. Therefore, the system described here should only be considered as a model system. However, we feel that this system provided new insights into the mechanisms by which 6-nitronorepinephrine can interact with the nervous system.

In conclusion, these data show that vasoactivities of 6-nitronorepinephrine involve α_1 adrenoceptors in the contraction being induced by 6-nitronorepinephrine alone, and H_2O_2 in endothelium-dependent-potentiation of the norepinephrine-induced contraction.

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