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Abstract

In the present study, we aimed to obtain further evidence in favour of the hypothesis that nitric oxide (NO) is a major mediator of endothelium-dependent vasorelaxation and to clarify whether NO plays a role in papaverine-induced vasorelaxation. The relaxant effects of acetylcholine (Ach), acidified NaNO2 or papaverine were investigated on isolated helical strips of the rat thoracic aorta precontracted with phenylephrine in an organ bath containing Krebs solution aerated with 95% O2 and 5% CO2. The relaxation was quantified as % peak reduction of phenylephrine contracture. Saponin abolished the relaxant effects of Ach completely whereas it had no effect on the responses to acidified NaNO2 or papaverine. NG-nitro-L-arginine (L-NOARG) reduced the effects of Ach significantly, but it was ineffective on the relaxation induced by acidified NaNO2. The inhibitory action of L-NOARG was partly restored by L-arginine, but not by D-arginine. Hemoglobin, hydroxocobalamin and hydroquinone exhibited significant inhibition on the relaxation evoked by Ach and acidified NaNO2. L-NOARG, hydroxocobalamin and hydroquinone caused only limited but significant decrease in the relaxation due to papaverine. This phenomenon was also observed by increasing phenylephrine concentration leading to an enhancement in the contraction. Our findings strongly support the view that Ach-induced relaxation of rat aorta strips is mediated by free NO released from the endothelium and the results suggest that NO may indirectly contribute to papaverine-induced relaxation.

KEYWORDS: rat aorta helical strips, nitric oxide, papaverine, hydroxocobalamin, hydroquinone

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Papaverine-Induced and Endothelium-Dependent Relaxation in the Isolated Rat Aortic Strip

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In the present study, we aimed to obtain further evidence in favour of the hypothesis that nitric oxide (NO) is a major mediator of endothelium-dependent vasorelaxation and to clarify whether NO plays a role in papaverine-induced vasorelaxation. The relaxant effects of acetylcholine (Ach), acidified $NaNO_2$ or papaverine were investigated on isolated helical strips of the rat thoracic aorta precontracted with phenylephrine in an organ bath containing Krebs solution aerated with 95% O_2 and 5% CO_2. The relaxation was quantified as % peak reduction of phenylephrine contracture. Saponin abolished the relaxant effects of Ach completely whereas it had no effect on the responses to acidified $NaNO_2$ or papaverine. N^G-nitro-L-arginine (L-NOARG) reduced the effects of Ach significantly, but it was ineffective on the relaxation induced by acidified $NaNO_2$. The inhibitory action of L-NOARG was partly restored by L-arginine, but not by D-arginine. Hemoglobin, hydroxocobalamin and hydroquinone exhibited significant inhibition on the relaxation evoked by Ach and acidified $NaNO_2$. L-NOARG, hydroxocobalamin and hydroquinone caused only limited but significant decrease in the relaxation due to papaverine. This phenomenon was also observed by increasing phenylephrine concentration leading to an enhancement in the contraction. Our findings strongly support the view that Ach-induced relaxation of rat aorta strips is mediated by free NO released from the endothelium and the results suggest that NO may indirectly contribute to papaverine-induced relaxation.

Key words: rat aorta helical strips, nitric oxide, papaverine, hydroxocobalamin, hydroquinone

Previous studies have suggested that nitric oxide (NO) or a closely related substance may be the main endothelium-derived relaxing factor (EDRF) in blood vessels. It has also been demonstrated that EDRF is generated exclusively from the metabolism of L-arginine (1, 2). This endogen substance regulates the tone of vascular smooth muscle and may play an important role in the control of blood pressure. On the other hand, acetylcholine (Ach) causes relaxation which is dependent on the presence of endothelium (3, 4). EDRF plays an essential role in mediating this kind of vasodilatation. Some substances, including organic nitrates, however, relax vascular tissues by an endothelium-independent mechanism (1).

In the present study, we aimed to obtain further evidence in favor of the hypothesis that NO or a related substance is a major mediator of endothelium-dependent relaxation. For this purpose, we tested whether the relaxation induced by Ach and acidified $NaNO_2$ in the isolated rat thoracic aorta could be inhibited by N^G-nitro-L-arginine (L-NOARG), hemoglobin (Hb), hydroxocobalamin (HC) or hydroquinone (HQ), all of which are known to inhibit the relaxant responses mediated by endothelium-dependent mechanisms of various vascular tissues (5-10). In addition, the effects of the same pharmacological substances on papaverine-elicited relaxation were evaluated since in a previous study on the rat gastric mucosal blood flow, a dose-dependent antagonism due to L-NOARG on the protective and hyperemic effects of papaverine against ethanol-induced damage in the gastric mucosa was observed (11). It might be interesting to investigate whether nitrergic mechanism plays any role in papaverine-induced relaxation, since papaverine is widely used as a reference agent to test selectivity of substances

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affecting nitrergic mechanism.

Materials and Methods

Male albino rats (Wistar strain) weighing 200 to 250 g were killed by a sharp blow to the neck (n = 80). Thoracic part of the aorta was carefully removed and placed in a Petri dish containing Krebs solution (composition [mM]; NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11). The vessel was dissected from the connective tissue and cut into helical strips (30 mm long and 2 mm wide) keeping the endothelium intact. The strips were then suspended vertically under 0.5 g tension in 40 ml organ baths containing Krebs solution which was continuously bubbled with 95 % O_2 and 5 % CO_2. Temperature was maintained at 37 °C. The preparations were allowed to equilibrate for 1 h. During this period they were washed with fresh Krebs solution at 15 min intervals. The responses were recorded by isotonic transducers (7006, Ugo Basile, Italy) on a polygraph (Gemini 7070, Ugo Basile).

After an equilibrium, period the tissue was treated with 0.05 μM phenylephrine resulting in an active tone that reached a steady state within 20 min, thereafter, Ach (1 μM), acidified $NaNO_2$ (10 μM) or papaverine (10 μM) was added to the bathing medium. The relaxation was monitored for 40 min and the tissue was washed with fresh Krebs solution. This procedure was repeated two times at 60-min intervals. Thus, two responses were recorded for each relaxant agent during a trial. In a series of experiments, after the first relaxation was recorded, the tissue was placed into Krebs solution containing L-NOARG (5, 10, 100 or 1,000 μM), L-arginine (0.5 mM), D-arginine (0.5 mM), Hb (1,5 or 10 μM), HC (5, 10, 30 or 100 μM) or HQ (0.1, 5, 8 or 10 μM) and the second relaxation was examined in the presence of these substances. In another series, the preparation was initially exposed to saponin (50 mg/l) for 60 min to remove the endothelium. At the end of the incubation period, saponin was washed out and the responsiveness of the tissue to relaxing agents was evaluated according to the protocol mentioned above, except that only one relaxation due to papaverine was recorded. In these experiments, Ach was also tried to confirm endothelium disruption. Some additional experiments were performed on the papaverine-induced relaxation so that the phenylephrine concentration (0.05 μM) used to obtain an active tone in all previous experiments was increased (to 0.1, 0.5 or 10 μM) prior to the second papaverine addition into the bathing medium. Thus, the action of the increment in the initial contraction force was evaluated on the relaxation produced by papaverine.

Drugs and solutions. Stock solutions of phenylephrine hydrochloride, Ach, HC and papaverine were dissolved in distilled water and stored at 4 °C. Saponin, L-NOARG, L-arginine, D-arginine, HQ were prepared daily in Krebs solutions. Acidified $NaNO_2$ was prepared in distilled water and stored at 4 °C. With the exception of $NaNO_2$ (Merck Chemical), all of the drugs were obtained from Sigma Chemical.

Statistical considerations. The relaxation was quantified as % peak reduction of phenylephrine contracture. The mean values of the first application and those of the second application series were calculated separately for each experimental group. All data were

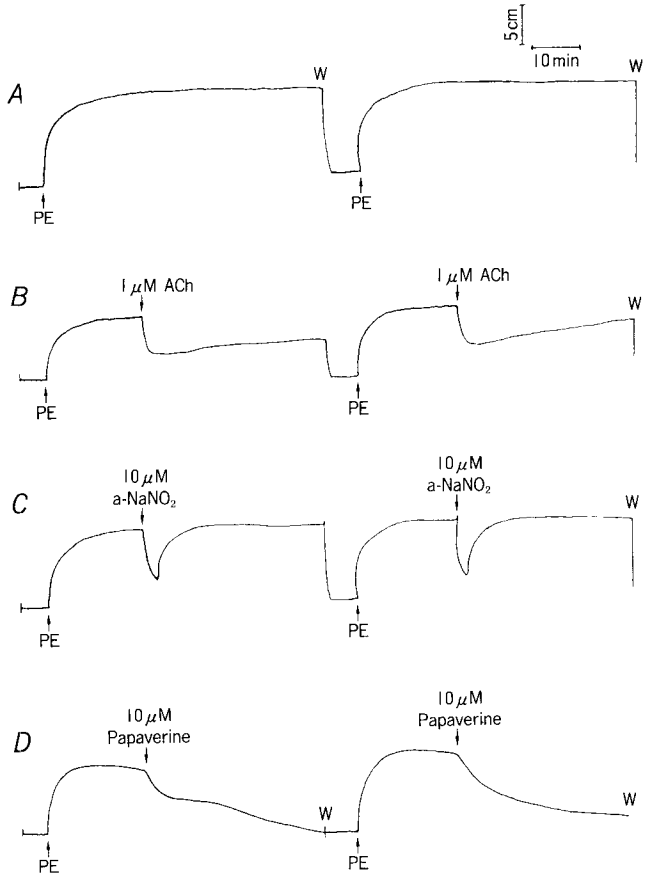

Fig. 1 Typical tracings showing (**A**) contracture induced by 0.05 μM phenylephrine (PE) and relaxant effects of (**B**) 1 μM acetylcholine (Ach), (**C**) 10 μM acidified $NaNO_2$ (a-$NaNO_2$) and (**D**) 10 μM papaverine on the rat aorta precontracted with 0.05 μM PE. W: Wash out

analyzed using the Wilcoxon matched-pairs signed-ranks test. P values of less than 0.05 were considered to be significant.

Results

Response of the tissue to phenylephrine. The drug (0.05 μM) caused a sustained contraction reaching a steady state within 10 min (Fig. 1A). Although the amplitude of the contraction increased slightly in response to the second application of the drug, its general profile did not change. The higher the phenylephrine concentration (0.1, 0.5 and 10 μM) applied to the bathing medium, the greater the tension observed.

Response to Ach or acidified $NaNO_2$. One μM Ach relaxed the tissue precontracted with 0.05 μM phenylephrine (Fig. 1B). The response was fast in onset, followed by a long lasting steady relaxation.

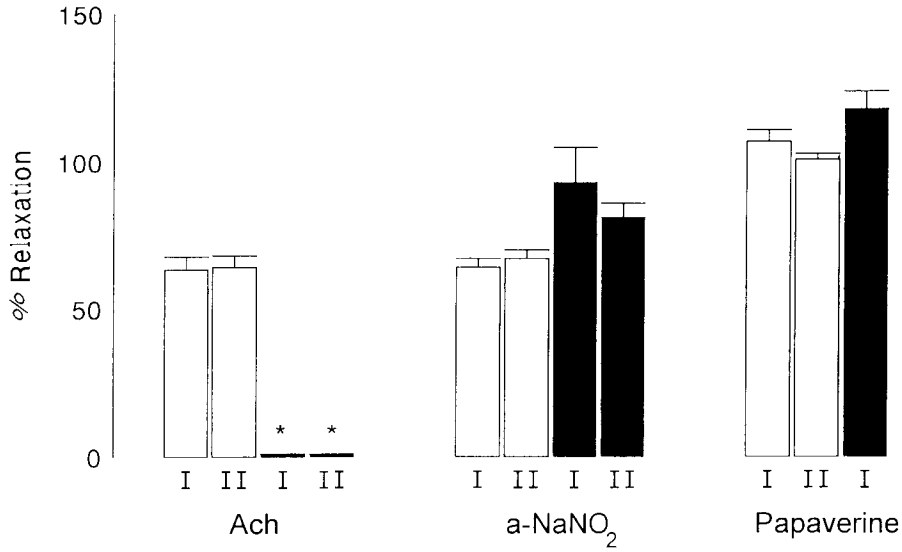

Fig. 2 Effects of saponin treatment (50 mg/L; solid bars) on relaxant actions of 1 μM acetylcholine (Ach), 10 μM acidified $NaNO_2$ (a-$NaNO_2$) or 10 μM papaverine on the rat aorta precontracted with 0.05 μM phenylephrine. Each column represents the mean relaxant response expressed as % of phenylephrine contracture. Open bars indicate control responses (controls were not treated with saponin). The vertical bars indicate standard errors. Numerals I and II show the first and second applications, respectively (n = 6–8).

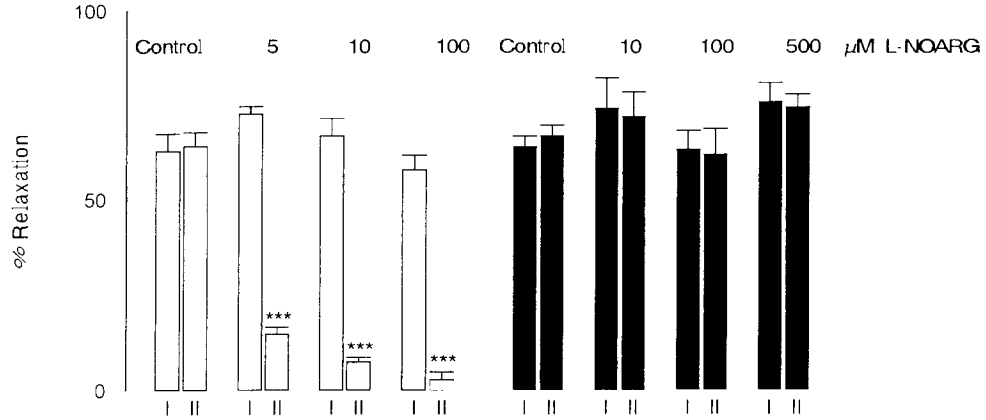

Fig. 3 Effects of N^G-nitro-L-arginine (L-NOARG) on relaxant actions of 1 μM acetylcholine (open bars) or 10 μM acidified $NaNO_2$ (solid bars) on the rat aorta precontracted with 0.05 μM phenylephrine. Each column represents the mean relaxant response expressed as % of phenylephrine contracture. The vertical bars indicate standard errors. Numerals I and II show the first and second applications, respectively (n = 6 8). ***$P < 0.005$.

However, some relaxant responses were biphasic in shape, *i.e.* relaxation followed by contraction. Ten μM acidified $NaNO_2$ elicited a rapid and transient relaxation (Fig. 1C). The mean values of the second application were not significantly different from those of the first.

Effect of saponin. Preincubation with saponin (50 mg/l) of the tissues for 1 h completely abolished the relaxant responses to Ach. However, the same substance was found to be ineffective on the relaxation induced by acidified $NaNO_2$ (Fig. 2).

Effects of L-NOARG, L-arginine or D-arginine. L-NOARG (5, 10 or 100 μM) inhibited the Ach-induced relaxation in a dose-dependent manner, but it did not affect the relaxation produced by acidified $NaNO_2$ (Fig. 3). L-NOARG raised baseline tension and augmented phenylephrine-induced contractions. However, the effect was not particularly noticeable and thus did not cause any problems throughout the experiment. The inhibition due to L-NOARG on Ach-induced relaxation

Table I The effects of N^G-nitro-L-arginine (L-NOARG), L-arginine or D-arginine on the relaxation induced by 1 μM acetylcholine

Control (n = 22)	106.9 ± 8.9
5 μM L-NOARG (n = 6)	19.8 ± 4.6**
500 μM L-arginine (n = 6)	94 ± 13.0
500 μM D-arginine (n = 6)	96.2 ± 14.7
5 μM L-NOARG + 500 μM L-arginine (n = 6)	71.4 ± 3.0*
5 μM L-NOARG + 500 μM D-arginine (n = 5)	6.4 ± 1.0**

*$P < 0.01$; **$P < 0.001$ (compared with control)
Values are presented as % of control relaxation (mean ± S.E.).

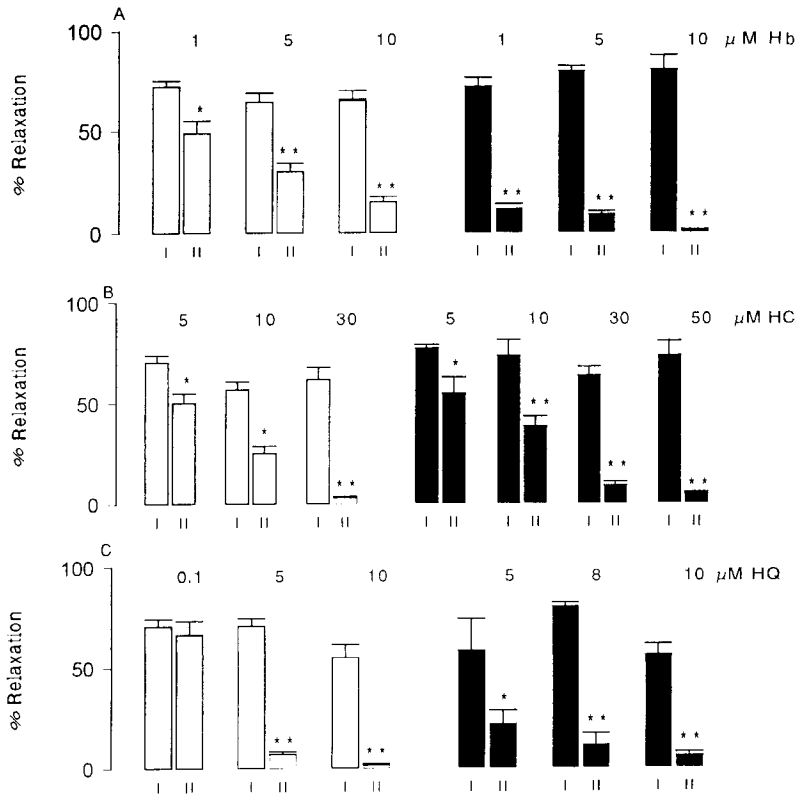

Fig. 4 Effects of (**A**) hemoglobin (Hb), (**B**) hydroxocobalamin (HC) or (**C**) hydroquinone (HQ) on relaxant actions of 1 μM acetylcholine (open bars) or 10 μM acidified $NaNO_2$ (solid bars) on the rat aorta precontracted with 0.05 μM phenylephrine. Each column represents the mean relaxant response expressed as % of phenylephrine contracture. The vertical bars indicate standard errors. Numerals I and II show the first and second applications, respectively (n = 6–8). *$P < 0.02$; **$P < 0.005$

could be restored by 0.5 mM L-arginine, but not by 0.5 mM D-arginine (Table 1). Neither L-arginine nor D-arginine affected the response to Ach.

Effects of Hb, HC or HQ. The relaxation in response to Ach or acidified $NaNO_2$ was significantly reduced by Hb (1, 5 or 10 μM), HC (5, 10, 30 or 50 μM) and HQ (0.1, 5 or 10 μM) (Fig. 4). In addition, the rate of development of relaxation produced by acidified $NaNO_2$ was decreased and the duration was prolonged. Hb, HC and HQ produced a concentration-dependent enhancement of phenylephrine contractions and caused an increase in the baseline tension.

Papaverine experiments. Ten μM papaverine frequently caused a biphasic response which consists of a relatively rapid phase followed by a slow sustained relaxation (Fig. 1D). Preincubation with saponin (50 mg/l) did not affect the full extent of the relaxant response to papaverine at the end of the monitoring time (Fig. 2). L-NOARG and HC caused a relatively small but significant decrease in the relaxation induced by papaverine (Figs. 5B and C), a finding which was observed for HQ as well (data not shown). All of these agents potentiated the vasoconstrictor response due to phenylephrine. The enhancement of the contraction with rising phenylephrine concentrations affected the relaxation to papaverine in a similar manner to that induced by L-NOARG and HC (Fig. 5A). In general, the higher the amount of phenylephrine given to the bath medium, the less relaxation induced by papaverine observed.

Discussion

The results of the present study suggest that free NO released from the endothelial cells may play a major role

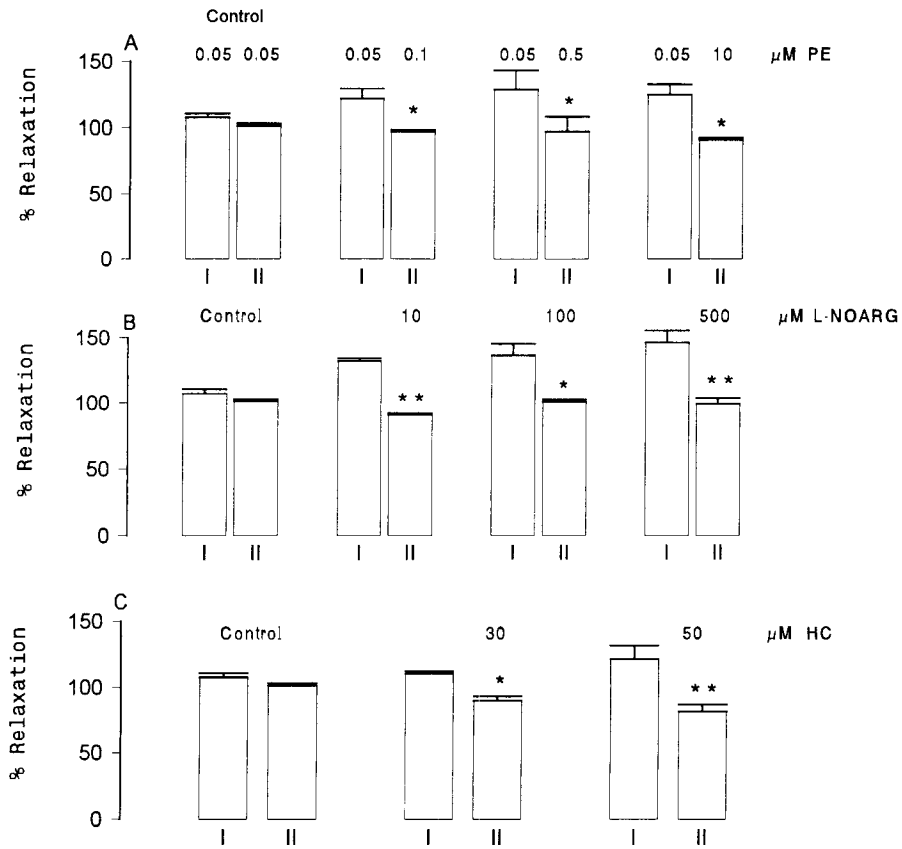

Fig. 5 Similarities between the effect of reinforced contraction produced by rising phenylephrine (PE) concentration (**A**) and N^G-nitro-L-arginine (L-NOARG) (**B**) or hydroxocobalamin (HC) on 10 μM papaverine induced relaxation. Each column represents the mean relaxant response expressed as % of phenylephrine contracture. The vertical bars indicate standard errors. Numerals I and II show the first and second applications, respectively (n = 6–8). $^*P < 0.05$; $^{**}P < 0.02$ (compared with the first application).

in Ach-induced relaxation, and it may indirectly, but not directly, contribute to papaverine-evoked relaxation in the rat thoracic aorta.

Phenylephrine elicited sustained contraction possibly mediated via α_1 adrenoceptors (12). Contractile activity has been found to be reproducible and regular in shape. Ach produced noticeable relaxation in endothelium-intact aortic strips, whereas it elicited no relaxation in the tissues preincubated with saponin (which has detergent activity and disrupts the endothelium). However, exogenously applied NO (present in the acidified solution of $NaNO_2$) produced relaxation in tissues treated with saponin. This result further supports the view that the endothelium is necessary for the production of relaxation by Ach (1, 3, 13).

Another finding is that the relaxation to Ach was significantly reduced or completely inhibited by L-NOARG. However, the relaxation induced by acidified $NaNO_2$ was not affected by L-NOARG. The antagonism was stereospecific; because responses inhibited by L-NOARG could be restored by L-arginine and not by D-arginine. Our findings lend further support to the idea that EDRF is synthesized from L-arginine by nitric oxide synthase (NOS) and that cholinergic relaxation of vascular smooth muscle is predominantly mediated by the release of this substance, as shown in previous studies (3-6, 13). L-NOARG affected basal tension, suggesting that there is continuous release of either free NO or NO-containing substances. A similar finding was also observed in the cerebral arteries in which NO regulate basal tone (14). On the other hand, the same substance enhanced the contraction induced by phenylephrine possibly due to an inhibition in EDRF release evoked by stimulation of α_2 adrenoceptors from the endothelial cells, as observed in large canine arteries (15) and human subcutaneous resistance vessels (16).

Hb, which is reported to sequester NO (7, 17), significantly reduced relaxation in response to Ach and acidified $NaNO_2$. This effect is due to the affinity of the heme group to NO (8). Likewise HC (vitamin B_{12}) which is thought to act as a NO scavenger (7, 9, 18) exhibited similar inhibition on the relaxation produced by Ach and acidified $NaNO_2$. HC reacts with NO to form nitrosocobalamin which may result in the inactivation of NO (9). These findings support the notion that cholinergic relaxation is mediated by endothelium-derived NO (3). In addition, HC caused enhancement in the baseline tension and response to phenylephrine in the similar manner as L-NOARG did. HQ, acting as free radical scavenger or superoxide anion generator (10), also affected the responses to both of Ach and acidified $NaNO_2$. Thus, the latter result also provides further support for the free NO hypothesis.

L-NOARG, HC and HQ caused partial inhibition of in the relaxation induced by papaverine. This phenomenon may result from strengthened contraction since suppression of NOS or scavenging of NO led to an increase in the initial contraction amplitude evoked by phenylephrine. In fact, a similar inhibition was observed in the papaverine-induced relaxation when the phenylephrine concentration was raised. Therefore, the effect may be indirect. This view is supported by the finding that saponin pretreatment was ineffective on the relaxation produced by papaverine, showing that the relaxation is not endothelium dependent.

In conclusion, the results of the present study indicate that the relaxation in response to Ach of isolated rat aortic strips is mediated by free NO released by endothelium. This mechanism may also be responsible for the very potent vasodilator effect of muscarinic substances in living animals, as previously suggested (2). However, our findings suggest that nitrergic mechanisms may contribute indirectly but not directly to the papaverine-induced relaxation in the rat thoracic aorta precontracted with phenylephrine.

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