L-Arginine의 산화질소생성과 무관한 혈관이완효과

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=Abstract=

Nitric Oxide/cGMP-Independent Vasorelaxation Enhanced by L-Arginine

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It has not been clear whether L-arginine plays solely a role contributing to vascular nitric oxide (NO) synthesis. To investigate the mechanisms by which L-arginine induces vasorelaxation, effects of L-arginine on the isometric tension, and tissue NOx and cyclic guanosine monophosphate(cGMP) contents were examined in the isolated rat thoracic aorta.

L-Arginine induced a dose-dependent relaxation of aortic rings only with intact endothelium only. The vasorelaxation response to low concentrations of L-arginine was abolished by the pretreatment with NG-nitro-L-arginine methyl ester(L-NAME, 10-4 mol/L), whereas the relaxation caused by higher concentrations L-arginine(10-5-10-3 mol/L) was maintained and even more pronounced in the presence of L-NAME. L-Arginine did not affect the vascular tension precontracted with KCl. The vascular tissue contents of NOx/cGMP were not significantly affected by L-arginine, while they were decreased by L-NAME. L-Arginine could not completely recover the NOx/cGMP decreased by L-NAME. Methylene blue only partially antagonized the relaxation response to L-arginine. Indomethacin did not affect the L-arginine-induced vasorelaxation, whereas ouabain markedly attenuated the relaxation. It is suggested that L-arginine induces vasorelaxation not only through its contribution to NO synthesis, but also through enhancing another endothelium-dependent mechanism which is NO/cGMP-independent and cyclooxygenase-independent.

(Korean J Thorac Cardiovasc Surg 1998;31:102-7)

Key words : 1. Vasodilation
2. Nitric oxide
3. Vasodilator agents
4. Aorta

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† This work was supported by a Research Grant from Korea Science and Engineering Foundation (97-04-03-09-3)
논문접수일 : 97년 1월 20일 심사통과일 : 97년 4월 1일

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INTRODUCTION

The contribution of nitric oxide(NO) to vasodilation in vivo has been demonstrated using inhibitors of NO synthesis, in which the pressor response to the inhibitors is reversed by a surplus of its precursor, L-arginine[1]. The direct role for L-arginine in normalizing the high blood pressure has been also known. In the healthy as well as in patients with essential hypertension, treatment with L-arginine causes a rapid reduction of systolic and diastolic blood pressures[2]. In addition, L-arginine may prevent the development of hypertension in animals prone to this disease[3]. Although vasodilator effects of L-arginine have been attributed to its contribution as a substrate for vascular NO synthesis[4], it is not clear whether L-arginine plays solely a role contributing to NO synthesis.

Several experiments have shown that prolonged incubation of isolated arteries uncovers relaxing effects of L-arginine[5,6]. It has been also reported that L-arginine induces a concentration- dependent relaxation of vascular rings only in the presence of NG-nitro-L-arginine methyl ester (L-NAME)[7]. The biological effects of L-arginine may thus be multifactorial, including its role to serve as an NO precursor.

The present study was aimed at investigating whether L-arginine contributes to the mechanism(s) which is not dependent on NO generation. Effects of treatment with L-arginine on the isometric tension, and vascular NOx and cGMP contents were determined in the isolated rat thoracic aorta in the absence and presence of L-NAME.

METHODS

Recording isometric tension of isolated thoracic aorta

Rat thoracic aortae were taken under thiopental anesthesia, and they were prepared into rings 5-mm long. Each ring was suspended in a tissue bath containing physiologic salt solution (PSS) at 37℃, while the solutions was continuously bubbled with 95% O_2-5% CO_2(pH 7.4). The composition(in mmol/L) of PSS used was NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and glucose 11.5.

The one end of the ring was fixed to the bottom of the bath and the other attached to an isometric force-displacement transducer(Grass FT03, Grass, USA). Baseline load placed on the ring was 2.0 g. Two to three rings were prepared from one animal and the results were averaged to provide a single rat datum.

The responses to L-arginine and sodium nitroprusside were examined in the aortic rings precontracted with EC_{80} phenylephrine(3.5×10^{-6} mol/L). The response was expressed as a percent change from the phenylephrine-induced maximum contraction. Drugs used were all purchased from Sigma Chemical Company, unless stated otherwise.

Inorganic NOx determination

The aortic ring was treated with L-NAME (10^{-4} mol/L) in vitro for 60 min. It was then subjected to precontraction by phenylephrine(3.5×10^{-6} mol/L) for 3 min, followed by addition of ACh(10^{-5} mol/L) or L-arginine(10^{-3} mol/L) for 30 sec. The reaction was terminated by a quick freeze. Each ring was homogenized in 0.5 mL of ice-cold absolute methanol, and allowed to stand at 4℃ for 18~20 h. Following centrifugation of the homogenate at 10,000×g for 10 min, the supernatant(300 μL) was used to determine the nitrite content. E. coli-derived nitrate reductase was used to reduce nitrate to nitrite, and the data were expressed as NOx. The remaining pellet was provided for protein quantification.

cGMP contents in vascular segments

Aortic rings were incubated in a 15 mL beaker at 37℃. PSS in the beaker was saturated with 95% O_2-5% CO_2(pH 7.4). A 2-h equilibration period was allowed to elapse. The aortic ring was treated with L-NAME(10^{-4} mol/L) for 60 min. It was then coincubated with L-arginine for 10 min, and quickly frozen to be stored at -70℃.

Frozen tissues were homogenized in 1 mL of 10% trichloroacetic acid at 4℃. The homogenate was centrifuged at 2,500×g for 30 min at 4℃, and the supernatant was separated from the pellet. The supernatant was extracted 4 times with 3 mL of water-saturated ether. The extract was then acetylated and assayed for cGMP using a radioimmunoassay kit (Amersham). The pellet was used for protein assay.

Statistics

Results are expressed as means±SEM. The statistical significance of differences between the groups was assessed by one-way analysis of variance followed by Bonferroni's test for multiple comparisons.

RESULTS

Vascular responses to L-arginine

Fig. 1 depicts the responses to L-arginine of the isolat-

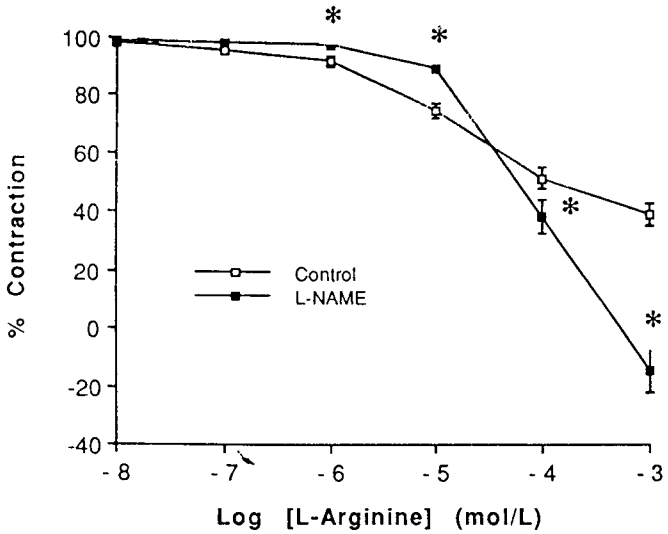

Fig. 1. Effects of L-arginine on the isometric tension of thoracic aortic rings with intact endothelium. L-NAME denotes the vascular responses in the presence of L-NAME (10-4 mol/L). n=Numbers of animals examined. A percent change from the maximum contraction obtained by phenylephrine ($3.5x10^{-6}$ mol/L) is depicted. *p<0.05, compared with control.

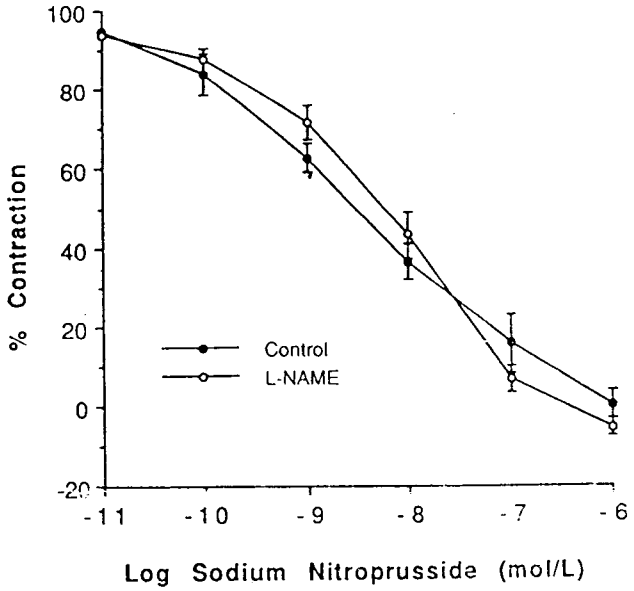

Fig. 2. Effects of sodium nitroprusside on the isometric tension of thoracic aortic rings in the absence and presence of L-NAME. Legends as in Fig. 1.

ed thoracic aorta. L-Arginine induced a dose-dependent relaxation of rings precontracted with phenylephrine(3.5×10^{-6} mol/L). The relaxation response to low concentrations of L-arginine was
abolished by L-NAME pretreatment(10^{-4} mol/L), whereas the magnitude of the relaxation in response to higher concentrations of L-arginine was more pronounced in the

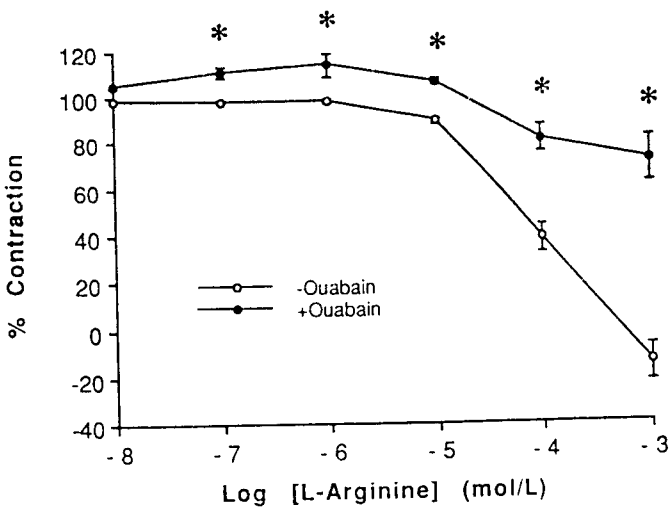

Fig. 3. Effects of ouabain on the relaxation response to L-arginine in the presence of L-NAME. Ouabain(10-5 mol/L) was treated with L-NAME(10^{-4} mol/L) 60 min before L-arginine was added. *p<0.05, compared with control.

presence of L-NAME.

Removal of the endothelium completely abolished the relaxation response to L-arginine(data not shown). The relaxation response to sodium nitroprusside was neither potentiated nor attenuated by the presence of L-NAME(Fig. 2). L-Arginine did not affect the vascular tension precontracted with KCl(3.5×10^{-2} mol/L) regardless of whether L-NAME was present or not (data not shown). Indomethacin(10^{-4} mol/L) did not affect the tension relaxed in response to L-arginine, whereas methylene blue(10^{-4} mol/L) only partially antagonized it (data not shown). On the other hand, ouabain markedly attenuated the relaxation response to L-arginine(Fig. 3).

Vascular NOx and cGMP contents

The vascular tissue contents of NOx and cGMP are shown in Fig. 4 and 5. L-NAME decreased the vascular contents of NOx and cGMP, whereas L-arginine did not significantly affect them. Nor was L-arginine effective in recovering the L-NAME-induced changes of NOx or cGMP contents.

DISCUSSION

L-Arginine has been shown to induce a concentration-dependent relaxation of vascular rings in the presence of L- NAME in the previous study[7]. Therefore, although L-arginine may produce a vasodilatory effect via stimulating synthesis and release of NO[4] , it is unclear whether it contributes to NO synthesis only. In the present study, we also found that the relaxation response to higher

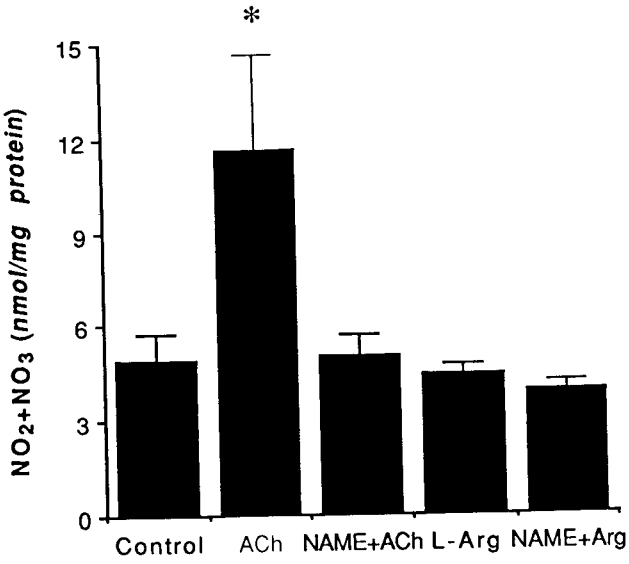

Fig. 4. Vascular tissue nitrite and nitrate contents. The aortic ring was treated with L-arginine(10^{-3} mol/L) in the absence and presence of L-NAME (10^{-4} mol/L for 60 min). The response to acetylcholine (ACh) is also presented as positive control. Number of rats in each column is 5.

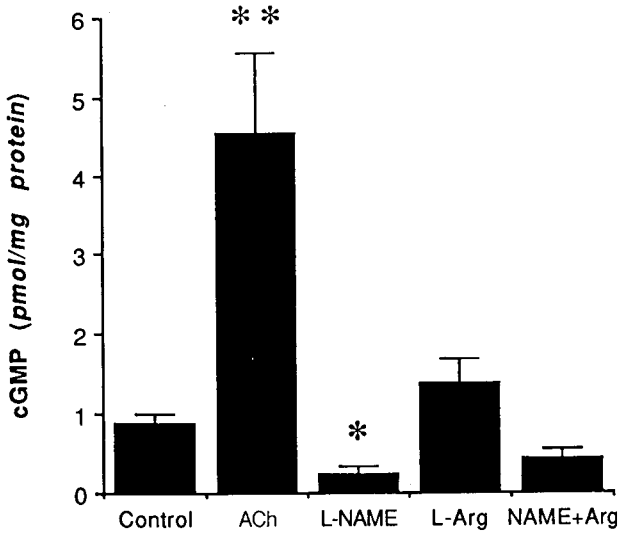

Fig. 5. Vascular tissue cGMP contents under control and stimulated conditions. The aortic ring was treated with either L-arginine (10-3 mol/L), L-NAME (10-4 mol/L), or both. Number of aortic rings in each column is 4-8. *p<0.05, **p<0.01; compared with control.

concentrations of L-arginine was more pronounced in the presence of L-NAME, whereas the relaxation response to low concentrations of L-arginine was abolished. The relaxation response to L-arginine in the presence of L-NAME may in fact reflect a reversal of the antagonistic effect of L-NAME on NO system. However, it is also clear that the relaxation response to L-arginine becomes uncovered or augmented in the presence of L-NAME. L-Arginine may produce vasodilators other than NO when its major contribution to NO synthesis is inhibited, such as in the presence of L-NAME. It is unlikely, however, that the augmented relaxation results from a hypersensitivity due to the inhibited NO activity, since the vasorelaxation in response to sodium nitroprusside was not potentiated by L-NAME pretreatment.

Our findings in the present study support an L-arginine-induced mechanism independent of NO/cGMP system in several ways. (1) L-Arginine(10^{-3} mol/L) in the presence of L-NAME decreased the vascular tension by 60% without increases in NOx and cGMP contents. (2) Methylene blue only partially restored the vascular tension when the vasculature was relaxed by L-arginine. (3) When the contractile tension of the L-arginine-relaxed vascular ring was resumed by L-NAME, it was preceded by a transient relaxation. Previous studies may also substantiate the speculation, in that NG-monomethyl- L-arginine (L-NMMA), another inhibitor of NO synthesis, relaxes canine intrapulmonary artery[8]. Moreover, although L-NMMA causes contraction of the aorta by superoxide anion synthesis and inhibition of NO synthesis, it also causes relaxation at higher concentrations than 10^{-3} mol/L through synthesis of citrulline-like substances[9].

It has been shown that, in cultured endothelial cells, L-arginine increases the release of NO only in cells previously deprived of L-arginine[10]. This finding may be accounted for by internal pools of amino acids which are depleted by a prolonged incubation and replaced by L-arginine administration. On the other hand, L-arginine-induced vasorelaxation has been found to be potentiated by increasing incubation time in the bath and is endothelium-independent[6,7]. However, the relaxation was observed only in endothelium-intact preparations in the present study. In addition, the incubation time did not affect the L-arginine-induced relaxation. The discrepancy may result from a multifactorial interplay, including different experimental protocols, which remains to be elucidated.

On the other hand, L-NMMA has been shown to antagonize amiloride(an inhibitor of Na+/H+ countertransport), dibutyryl cAMP(to increase intracellular cAMP contents), and N-α-benzoyl L-arginine ethyl ester(to increase intracelluar cGMP contents)[9]. It is also known as antagonists of Na^+, K^+-ATPase[10]. These suggest that the activity of the substance(s) stimulated by L-arginine can be associated with modulation of various processes including Na^+/H^+ countertransport, recruitment of

intracellular cAMP or cGMP, or Na^+,K^+-ATPase activity.

Indeed, L-arginine-induced vasorelaxation was significantly attenuated by pretreatment with ouabain in the present study. It has been previously found that ouabain does not modify the relaxations induced by NO[11]. Therefore, the results obtained in the presence of ouabain cannot be attributed to NO but to be a mechanism other than NO. The factor responsible for the relaxation may be Na,K-ATPase activator. The previous finding that L-arginine restores Na^+, K^+-ATPase activity of hindlimb artery of rabbit, in which Na^+, K^+-ATPase activity was inhibited by hyperglycemia pretreatment[12], also supports such a notion. This Na,K-ATPase activator may result in a vasorelaxation through changing membrane potentials.

On the contrary, the inhibition of electrogenic Na,K-pump by ouabain induces membrane depolarization[13]. The depolarization may antagonize a hyperpolarization. To further support the speculation, KCl-induced contraction was not affected by L-arginine. This finding suggests that KCl-induced depolarization may antagonize a hyperpolarization, if any. However, the factor has to be differentiated from endothelium-derived hyperpolarizing factor (EDHF), since EDHF-induced vasorelaxation is neither affected by methylene blue nor is ouabain- sensitive[14]. It is also unlikely that the relaxation mediated by a cyclooxygenase-dependent mechanism, since it was not affected by indomethacin.

In summary, NO/cGMP-independent, but L-arginine-induced vasorelaxation has following features. First, it is endothelium- dependent. Second, the factor responsible for the relaxation is not a cyclooxygenase product but is associated with an enhanced activity of Na,K-ATPase. Third, it may be suppressed by NO under a basal condition, and is activated only when the normal activity of NO/cGMP pathway is attenuated. It is concluded that L-arginine induces vasorelaxation not only through its contribution to basal NO synthesis, but also through an activation of "Na,K-ATPase activator".

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=국문초록=

L-Arginine이 산화질소 생성의 전구물질로서 공헌하는 것 이외에 다른 기전에 의하여도 혈관이완을 일으키는가 구명하기 위하여 적출 흰쥐 흉부대동맥 표본에서 L-arginine에 의한 장력, 조직 산화질소 및 cGMP 함량 변동 등을 조사하여 다음과 같은 결과를 얻었다.

1. Phenylephrine(3.5×10^{-6} mol/L) 수축 대동맥 표본은 L-arginine($10^{-9}\sim10^{-3}$ mol/L)에 의해 용량의존 이완되었다. N^G-Nitro-L-arginine methyl ester(L-NAME, 10^{-5} mol/L) 전처치에 의해 저농도 L-arginine($10^{-9}\sim10^{-6}$ mol/L)에 혈관이완 효과는 소실되었으나 고농도 L-arginine($10^{-4}\sim10^{-3}$ mol/L)의 이완효과는 도리어 증강되었다. 내피층 파괴 혈관 표본은 L-arginine에 대해 이완반응을 보이지 않았다.
2. L-NAME(10^{-5} mol/L) 존재하에 일어나는 L-arginine 이완효과는 indomethacin 전처치에 의해 영향받지 않으나, ouabain 전처치에 의해 유의하게 감약되었다. 또한 L-arginine 이완효과는 methylene blue에 의해 부분적으로 길항되었다. KCl(3.5×10^{-2} mol/L) 수축 대동맥 표본은 L-arginine($10^{-9}\sim10^{-3}$ mol/L)에 의해 L-NAME (10^{-5} mol/L) 처치와 무관하게 이완반응을 보이지 않았다.
3. L-NAME는 혈관조직 산화질소 함량을 감소시키며 이 감소효과는 L-arginine(10^{-4} mol/L)에 의해 영향받지 않았다. 또한 L-NAME는 혈관조직 cGMP 함량을 감소시키나 이 감소효과는 L-arginine에 의해 영향받지 않았다.

이상의 실험성적은 L-arginine이 내피세포의 산화질소 및 cGMP 생성과 무관한 기전을 통해서도 내피의존 혈관이완효과를 나타냄을 시사하였다.