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EVIDENCE OF L-ARGININE/NITRIC OXIDE PATHWAY IN ENDOTHELIUM AND SMOOTH MUSCLE OF HUMAN INTERNAL MAMMARY ARTERY

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SUMMARY The L-arginine-nitric oxide(NO) pathway was investigated in human internal mammary artery (HIMA) in vitro. HIMA rings were mounted in organ bath, and then incubated in Krebs buffer for 1 to 8 hrs, relaxing agents were tested. Under these conditions, L-arginine (0.1 μM - 1 mM) elicited only minor relaxation after 2 hr incubation, whereas with increased incubation time(4,6,8 hrs), the concentration-dependent relaxation to L-arginine increased significantly in endothelium-intact and -denuded vessels. N^G-nitro-L-arginine (100 μM) or N^G-monomethyl-L-arginine (100 μM) or methylene blue (2.7 μM) partially inhibited L-arginine relaxation. In endothelium-intact HIMA and in both types of rings A23187(10 μM) and L-arginine(100 μM), respectively, increased the concentration of NO in medium and cGMP content of vascular tissues. These increases were partially inhibited by N^G-nitro-L-arginine (100 μM) or methylene blue (2.7 μM). Conclusion: in smooth muscle of HIMA L-arginine-NO conversion is calcium independent, which is different from that in endothelium.

The L-arginine-nitric oxide (NO) pathway plays a newly defined role in regulating vascular tone(1). NO may constitute, at least in part, the endothelium-derived relaxing factor (EDRF) (2, 3). Also, in smooth muscle of bovine pulmonary artery (4) and rat aorta (5), after prolonged incubation in buffer solution exogenous repletion of L-arginine enables maintenance NO formation or relaxation. However, conflicting results have been reported concerning the site of conversion of L-arginine to NO in blood vessel wall, since possible locations include either the endothelial cell layer alone (2), or the vascular smooth muscle myocytes alone (6), or both cell types (5, 7).

Human internal mammary artery (HIMA) has gained a prominent role as a donor vessel in coronary artery bypass surgery. However, HIMA is susceptible to spasm due to its small lumen, which is prone to constriction by vasoactive factors (8). Furthermore, surgical manipulation may impair the integrity of endothelial layer of a coronary graft. Therefore the identification of vasodilatory mechanisms is essential for understanding the regulation of HIMA reactivity and for improving its patency as a graft. Thus, the objective of the study was to identify the cellular site and mechanism of L-arginine relaxation in HIMA.

MATERIAL AND METHODS

Chemicals The following drugs were used: L-arginine hydrochloride, N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine(L-NOARG), acetylcholine

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(ACh), A23187, methylene blue (MB), norepinephrine (NE), indomethacin, sulfanilamide, N-(1-naphthyl)-ethylenediamine, trichloroacetic acid, streptomycin, penicilline (all Sigma), Sin-1 (a gift from Hoeschst Laboratories, Paris, France). A23187 was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO was less than 0.4%. Indomethacin was prepared in an equimolar (100 mM) concentration of sodium carbonate and then diluted with distilled water when necessary. Sin-1 was prepared in 5% glucose and used in the dark.

Organ bath experiments HIMA from 22 men and 2 women (age 47-75; mean 56±9 yrs) were obtained during coronary bypass surgery. The samples were not treated with pharmacological agents. Light microscopy, using silver nitrate, hematoxylin-eosin or Weigert's staining, respectively, rejected two cases of subintima fibrosis in the HIMA sample from corresponding experiments. HIMA endothelium was removed mechanically in some segments when necessary. Less than 2 hrs after tissue collection, the vessel rings(1.5-1.8 mm diameter) were suspended vertically in organ baths (10 ml, 37°C, bubbled with a 5% CO_2 -95% O_2 gas mixture) containing Krebs solution with composition (mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $MgSO_4$ 1.2; KH_2PO_4 1; $CaCl_2$ 2.5; EDTA 0.03; glucose 11.1 and 100 U/ml of both streptomycin and penicilline(10,11). Krebs solution was filter-sterilized. After equilibration at 1.5g tension for 3 hrs, ACh(1 nM-10 μM) relaxation test on NE(1 μM) precontraction (failure to relax was the evidence of adequate endothelial removal) and rinse for 2.5 hrs, indomethacin (10 μM) was added to Krebs solution. This step, 7.5 hrs far from vessel excision, was considered to be t=0 hr(5). Then preparations were incubated in Krebs solution with indomethacin (10 μM). After various incubation periods (t=1,2,4,6,8 hrs), the HIMA preparations were precontracted with NE (1 μM) and tested for responsiveness to vasodilating agents in a cumulative fashion. Inhibitors,when used, were added to Krebs solution 30 mins before NE stimulation after t=8 hr incubation(5).

Measurement of NO Nitrite was estimated in the medium surrounding the vasculature, as reported by Green et al(9) and by Wood et al(4). Briefly, before and after incubation as described above, HIMA preparations were stimulated with NE (1 μM) for 2 mins, and quickly transferred to a microtube containing 0.5 ml of Krebs solution with L-arginine (100 μM) and EDTA (5 mM). Then, this microtube was frozen quickly in liquid nitrogen (based on optimum response of preliminary time course, data not shown). Inhibitors, when used, were added to the medium 30 mins before NE stimulation. This 0.5 ml of media was mixed with 0.5 ml of reagent containing 0.5% sulfanilamide and 0.05% N-(1-naphthyl)-ethylenediamine. After 10 mins at 20°C, this 1 ml aliquot was measured at a wavelength of 550 nm. Concentrations were calculated according to a standard curve obtained in sodium nitrite solution.

Assay of cGMP Before and after incubation, HIMA had been precontracted, transferred to a microtube containing L-arginine (100 μM) and quickly frozen as described above (based on optimum response of preliminary time course, data not shown). Inhibitors, when used, were added to the medium 30 mins before NE(1 uM) stimulation. The preparations were homogenized in ice-cold 6% trichloroacetic acid. The homogenates were centrifuged at 1700 g for 10 mins at 4°C, and the supernatants were extracted with 3 volumes of water-saturated ether. cGMP was measured by radioimmunoassay (12) with a commercial kit (Amersham, France; BTI, MA, USA). The cGMP content was normalized to tissue protein which was determined by the method of Lowry et al(11).

Data analysis Data were expressed as mean ± SEM for n separate preparations. Contractile responses were expressed as force in g, relaxations as percentage of NE (1 μM) precontraction and the pD_2 value as the negative logarithm of ED_{50}. Data were analysed by one-way or two-way ANOVA followed by Duncan Multiple Range test, or unpaired Student's t test. A p value <0.05 was considered to be significant.

RESULTS

Organ bath experiments After t=1 and t=8 NE (1 μM) elicited no significantly changed precontractions, and Sin-1(30nM-30μM) elicited no significantly changed relaxations in dose-dependent fashion in both types of rings, respectively (n=5 each) [data not shown].

Very minor or no relaxation occurred to L-arginine (0.1 μM - 1 mM) in both types of rings after t=1 and/or t=2. With increased incubation periods of t=2 to t=8 in endothelium-intact or of t=4 to t=8 in endothelium-denuded preparations, a concentration-dependent relaxation to L-arginine was observed, which increased proportional to incubation time. After t=8, maximal relaxations of 95±6% and 90±5% were observed in endothelium-intact [Figure 1A] and -denuded preparations-

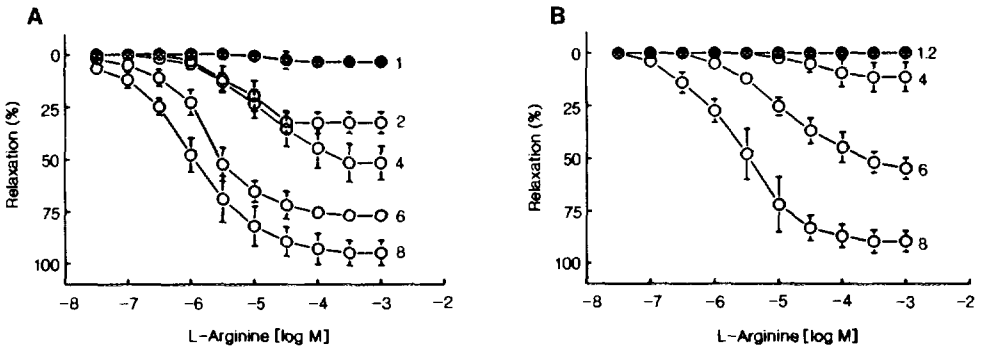

Figure 1. Concentration- and time-dependent relaxation curves in human internal mammary artery (HIMA) elicited by L-arginine.
The segments of HIMA were incubated in Krebs solution for *t*=1, 2, 4, 6, 8 hrs before being precontracted by norepinephrine (NE, 1 μM). (●): relaxation after *t*=1 hr incubation, used as a control. The numbers attached to curves refer to the various incubation periods in hr. **A**: Relaxation of endothelium-intact HIMA. **B**: Relaxation of endothelium-denuded HIMA. Each point is the mean of 5 separate experiments with SEM.

(*n*=5 each) [Figure 1B], with pD_2 values of 5.49±0.16 and 5.11±0.26, respectively. The relaxations to L-arginine after *t*=2 to *t*=8 were greater in endothelium-intact preparations. In contrast, in both endothelium-intact and -denuded preparations, only minor relaxations to L-arginine occurred after *t*=8 when the incubation medium contained L-arginine (1 mM) from the start (n=4 each) - [data not shown].

L-NOARG (100 μM) significantly reduced the maximal relaxations from 95±6% to 52±12% in endothelium-intact and from 90±5% to 45±9% in endothelium-denuded preparations (n=5 each) [Figure 2A,B]. Similarly, in endothelium-intact preparations, maximal relaxations were reduced from 95±6% to 69±6%, and from 95±6% to 25±11%, in the presence of L-NMMA(100 μM) or MB(2.7 μM), respectively

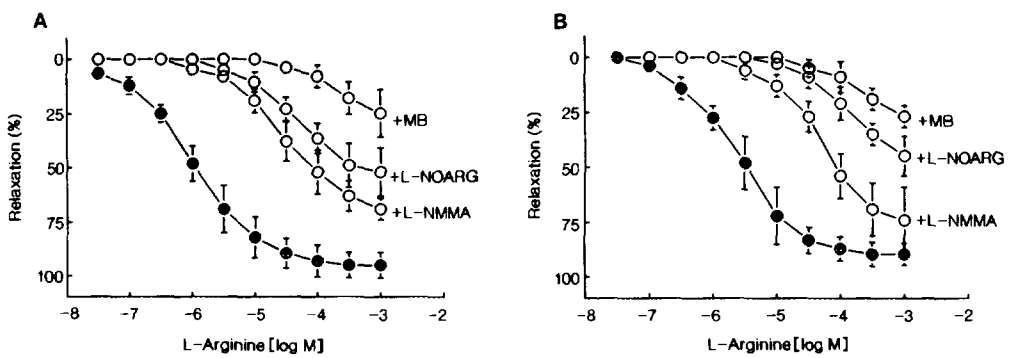

Figure 2. Effect of N^G-nitro-L-arginine (L-NOARG, 100 μM), N^G-monomethyl-L-arginine (L-NMMA, 100 μM) or methylene blue (MB, 2.7 μM) on L-arginine relaxation curves in human internal mammary artery(HIMA).
The segments of HIMA were incubated in Krebs solution for *t*=8 hrs before being precontracted by norepinephrine (NE, 1 μM). (●): Relaxation in the absence of inhibitor used as a control. (**A**) Endothelium-intact HIMA and (**B**) Endothelium-denuded HIMA. Each point is the mean of 5 separate experiments with SEM.

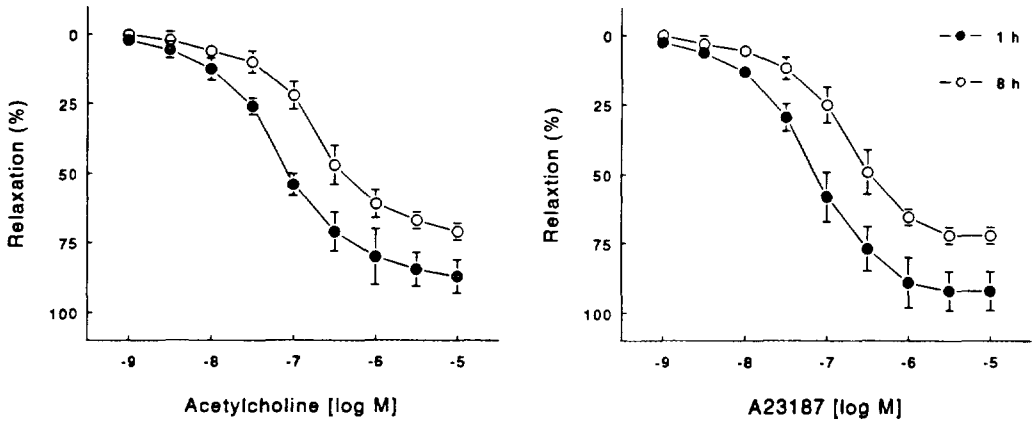

Figure 3. **Concentration-dependent relaxation curves in endothelium-intact human internal mammary artery (HIMA) elicited by Acetylcholine and A23187.** The segments of HIMA were incubated in Krebs solution for t=1 or t=8 hrs before being precontracted by norepinephrine (NE, 1 μM) . (●): Relaxation after t=1 incubation, used as a control. (○): Relaxation after t=8 incubation. (Up) acetylcholine; (Down) A23187. Each point is the mean of 4-5 separate experiments with SEM.

(n=5 each) [Figure 2A]. Also, in endothelium-denuded rings the maximal relaxations were reduced from 90±5% to 74±15%, and from 90±5% to 27±6% in the presence of L-NMMA(100 μM) or MB(2.7 μM), respectively (n=5 each) [Figure 2B].

ACh elicited concentration- and endothelium-dependent relaxation in arteries. However, this curve was shifted significantly to the right after t=8: pD_2 values were 7.27±0.06 after t=1 versus 6.73±0.11 after t=8 (n=5 each) [Figure 3A]. A23187 (1 nM - 10 μM) elicited relaxation only in endothelium-intact arteries. This curve, after t=8, was shifted to the right: pD_2 value was 7.20±0.05 after t=1 versus 6.77±0.06 after t=8 (n=4 each) [Figure 3B].

Modulation of NO concentration NO concentration, in control t=0, of 1.60+0.32 and 1.48+0.44 nmol/ml in endothelium-intact and -denuded rings were measured. After t=8, in control NO values were 0.84±0.05 and 0.79±0.09 nmol/ml in endothelium-intact and -denuded rings. L-arginine(100 μM) increased NO concentration to 3.56±0.32 and 2.90±0.54 nmol/ml in endothelium-intact and -denuded HIMA, respectively (n=4 each). After t=8, A23187 (10 μM) also increased NO value to 2.85±0.08 nmol/ml in endothelium-intact , but not in endothelium-denuded arteries (n=4 each). Exposure to L-NOARG (100 μM) decreased NO values from 3.56±0.32 to 1.25+0.27 nmol/ml in endothelium-intact and from 2.90±0.54 to 1.10+0.48 nmol/ml in endothelium-denuded preparations, respectively (n=4 each) [Figure 4].

Modulation of cGMP content cGMP content, in control t=0, of 4.80+0.50 pmol/mg protein and 3.10+0.45 pmol/mg protein in endothelium-intact and -denuded preparations were measured. After t=8, in control cGMP values were 3.72±0.24 and 1.90±0.25 pmol/mg protein in endothelium-intact and -denuded rings, and increased to 18.54±0.48 and 14.36±0.75 pmol/mg protein , respectively (n=4 each), when L-arginine(100 μM) was applied. The basal and increased cGMP contents after L-arginine application were greater in endothelium-intact than in -denuded HIMA. In endothelium-intact arteries, A23187(10 μM) also increased cGMP content to

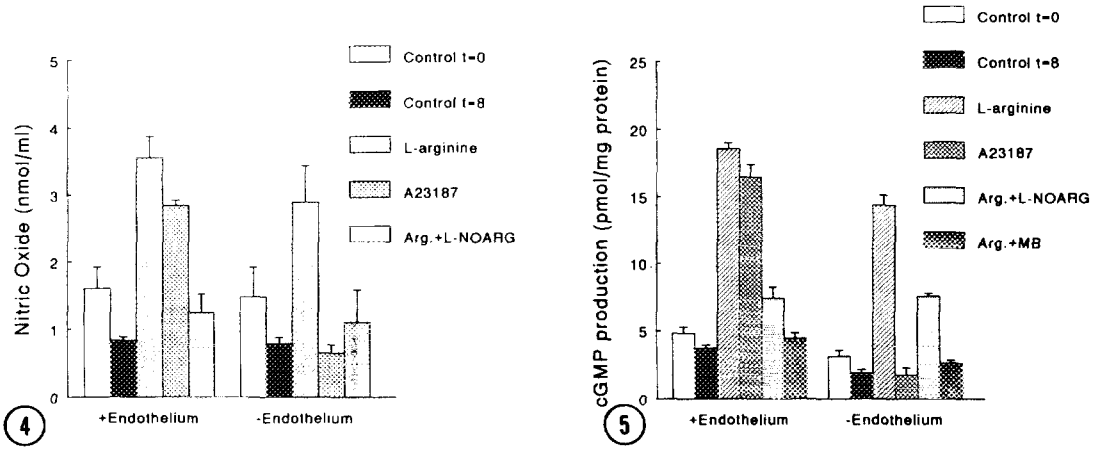

Figure 4. NO concentrations in the medium, compared in controls, in A23187 (10 μM)-evoked and in L-arginine (100 μM)-evoked with or without the presence of N^G-nitro-L-arginine (L-NOARG, 100 μM) in human internal mammary artery (HIMA). The segments of HIMA were incubated in Krebs solution for *t*=0 or *t*=8 hrs before being stimulated by norepinephorine(1 μM). Results are the mean of 4 separate experiments with SEM.

Figure 5. cGMP contents in controls and after incubation when the application of L-arginine (100 μM), A23187, N^G-nitro-L-arginine(L-NOARG, 100 μM) or methylene blue (MB, 2.7 μM) on L-arginine (100 μM)-evoked cGMP formation in human internal mammary artery (HIMA).
The segments of HIMA were incubated in Krebs solution for *t*=0 or *t*=8 hrs before being stimulated by norepinephrine (1 μM). Results are the mean of 4 separate experiments with SEM.

16.44±0.95 pmol/mg protein after *t*=8, compared to a control value of 1.73±0.55 pmol/mg protein (n=4 each), but not in endothelium-denuded preparations. In the presence of L-NOARG(100 μM) the corresponding cGMP values were 7.40±0.82 and 7.54±0.22 pmol/mg protein (n=4 each). Similarly, after MB(2.7 μM) exposure, cGMP contents were 4.45±0.40 and 2.63±0.21 pmol/mg protein, in endothelium-intact and -denuded rings, respectively (n=4 each) [Figure 5].

DISCUSSION

Our principal finding is that the magnitude of the relaxation elicited by L-arginine increased as a function of incubation time in both endothelium-intact and -denuded HIMA, associated with an increase in the modulation of NO and cGMP. One likely explanation for these is a gradual and relative depletion of L-arginine in the isolated blood vessel (4). Gold et al have demonstrated that the L-arginine content in fresh preparations is initially high and declines several-fold after prolonged incubation (14). Our results and those of Schini and Vanhoutte (5) concur with the depletion explanation by showing that incubation with L-arginine (1 mM) abolishes the relaxation to this amino acid. Multiple control experiments, such as no significantly changed NE precontractions, Sin-1 relaxations and blunted ACh, A23187 relaxations after *t*=1 and *t*=8, suggest that altered L-arginine mechanisms, rather than experimental situation, account for the time-dependent potentiation of L-arginine relaxation.

As expected, A23187, which specifically activates calcium dependent NO synthesis in endothelial cells, increased the modulation of NO only in endothelium-intact HIMA. However, both endothelium-intact and -denuded HIMA

possess the capacity to generate a relaxing factor in response to L-arginine administration. This factor has the chemical properties of NO, or a nitroso compound which decomposes spontaneously to liberate NO. The smooth muscle cells of HIMA were able to generate NO is confirmed by no morphologically or functionally identifiable endothelial cells present in the endothelium-denuded - preparations. These results are consistent with those of Wood et al(4) in endothelium-denuded bovine pulmonary arteries, and of Bernhart et al(15) in endothelium-denuded human mesenteric arteries. It deserves emphasis that L-arginine is relatively, instead of completely, depleted in our study. Further evidence is that pretreatment with L-NOARG inhibited markedly NO formation in both types of arteries. On the convertion from L-arginine to NO in vascular wall, constitutive and inducible formes of NO synthases, have been characterized. Constitutive NO synthase exists only in endothelium(11,16-20) and inducible NO isoenzyme is described in endothelial cells and smooth muscle cells(11,21-24). Our another recent observation further reveals(25) that calmidazolim impairs this L-arginine relaxation and decreases cGMP content in endothelial cells but not in smooth muscle cells in HIMA. This is the further evidence that the effect of calcium-calmodulin complex in constitutive synthase but not in inducible isoenzyme in HIMA. Inducible NO synthase in smooth muscle cells is induced by endotoxin in rat thoracic aorta in L-arginine depletion model(26), our present study, however, excludes this possibility due to antiendotoxin elements in the bath solution(10,11,16,27).

In our study, the content of cGMP which is implicated as second messenger, increased in response to L-arginine stimulation. Inhibitors of NO formation, L-NMMA and L-NOARG which partially inhibited L-arginine relaxation, NO formation, and cGMP modulation in both types of preparations, presumably possess a direct action on the L-arginine-NO pathway (28), since it was comparable between endothelium-intact and -denuded HIMA segments. This may suggest that L-arginine-NO conversion in endothelial cells and smooth muscle cells have different properties. Besides a increased basal vascular tone and/or an inhibition of guanylate cyclase(3, 29), MB also inhibits the conversion of L-arginine to L-citrulline (17), suggesting that L-citrulline is not a contributing vasodilator in our preparations. Furhtermore, MB has been recently reported to inhibit NO synthase at low concentration(30).

In conclusion, besides the L-arginine-NO pathway in endothelial cells, human vascular smooth muscle may have the necessary intracellular components to convert L-arginine to NO, which has different properties from that of endothelium. L-arginine-NO conversion is calcium independent. The physiological and clinical significance of a muscle-specific L-arginine-NO pathway in HIMA may be considerable, since endothelial layer impairment in a grafted vessel may occur after coronary bypass surgery(31,32). In view of this risk, the L-arginine-NO pathway in the vascular muscle cells may represent a potential target for vasodilator agents.

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