Effects of L- N^G -Monomethyl Arginine on the Cyclic GMP Formations in Rat Mesenteric Arteries

Noboru Fukuda, Yoichi Izumi, Masayoshi Soma, Yoshiyasu Watanabe,
Masashi Watanabe and Michinobu Hatano

*The 2nd Department of Internal Medicine, Nihon University School of Medicine,
Ooyaguchi-kami 30-1, Itabashi-ku, Tokyo 173, Japan*

Received June 3, 1991 Accepted October 14, 1991

ABSTRACT—To evaluate the contribution of L-arginine as a precursor of the endothelium-derived relaxing factor (EDRF) on vascular cyclic GMP formation, we examined the effects of L- N^G -monomethyl arginine (L-NMMA), and analog of L-arginine, on basal and acetylcholine (ACh)-, sodium nitroprusside (SNP)- and atrial natriuretic peptide (ANP)-induced cyclic GMP formations in rat mesenteric arteries. The mesenteric arteries were perfused with Krebs-Henseleit solution containing 0.2 mM isobutyl methyl xanthine. The effluents from the arteries were collected before and after infusions of graded doses of ACh, SNP or ANP in the absence or presence of 100 μ M L-NMMA, and the levels of cyclic GMP were measured. Basal and ACh-induced cyclic GMP formations in the mesenteric arteries were significantly inhibited in the presence of L-NMMA, whereas a concomitant infusion of 300 μ M L-arginine restored the inhibition of basal as well as ACh-induced cyclic GMP formations. L-NMMA did not affect SNP- and ANP-stimulated cyclic GMP formations, respectively. These results suggest that L-arginine is necessary for not only the stimulated cyclic GMP formation but also the basal cyclic GMP formation in the mesenteric arteries, whereas the SNP- and ANP-stimulated cyclic GMP formations in the arteries are independent of L-arginine.

Physiological roles of cyclic GMP in the regulation of vascular tonus have become apparent recently. The conversion of guanosine triphosphate to cyclic GMP in the vascular smooth muscle is catalyzed by two isoenzymic forms of guanylate cyclase, soluble and particulate guanylate cyclases (1). The soluble isoenzyme can be activated by free radicals such as nitric oxide (NO), including vasodilator-like nitrates and sodium nitroprusside (SNP) (2, 3), and porphyrins (4), whereas the particulate isoenzyme can be activated by atrial natriuretic peptide (ANP) (5).

On the other hand, vascular relaxation induced by acetylcholine (ACh), histamine,

bradykinin, Ca^{2+} ionophore A23187, thrombin or ATP is dependent on the existence of an intact endothelium (6, 7). These agents interact with specific receptors on the endothelium to induce the formation of endothelium-derived relaxing factor (EDRF) (6, 7). It has been established that EDRF stimulates soluble guanylate cyclase of the vascular smooth muscle, resulting in an increase in the levels of cyclic GMP (8, 9).

One of EDRFs has been identified chemically as NO (10). Macrophages (11) and endothelial cells in culture (12, 13) have been reported to synthesize NO from the terminal guanido nitrogen atom(s) of the amino acid L-

arginine. It has recently been found that the synthesis of NO and the vasodilating effects of EDRF are inhibited by L-N^G-monomethyl arginine (L-NMMA), an analog of L-arginine (14, 15).

Since vascular cyclic GMP formation is not equivalent to EDRF formation, we investigated the effects of L-NMMA on basal and ACh-induced cyclic GMP formations as compared with the effects on SNP- and ANP-induced cyclic GMP formations which are independent of EDRF, in order to evaluate the contribution of L-arginine to vascular cyclic GMP formations.

MATERIALS AND METHODS

Male Wistar rats weighing 200–300 g were used. Preparations of rat mesenteric arteries were made as reported previously (16). The rats were anesthetized with pentobarbital i.p., and the superior mesenteric arteries were cannulated 0.7-cm distal to the aorta. The vessels of the mesenteric arterial bed were separated close to the intestine and perfused at 3 ml/min using an infusion pump (MP-3, Tokyo Rikakikai, Japan) with Krebs-Henseleit solution that was gassed with 5% CO₂ in O₂ and had the following composition: 7.16 g/l NaCl, 0.32 g/l KCl, 0.2 g/l MgCl₂·6H₂O, 0.37 g/l CaCl₂·2H₂O, 0.12 g/l NaH₂PO₄, 2.1 g/l NaHCO₃ and 1.0 g/l dextrose. The mesenteric arteries were preperfused for over 60 min. Before every experiment, we tested 10⁻⁷ M ACh-induced vasorelaxations of the arteries precontracted with 10⁻⁵ M phenylephrine to confirm the presence of an intact endothelium. ACh, SNP, ANP or L-arginine was infused at 1.8 ml/hr via a side arm of the arterial cannula employing a micro-infusion pump (SP-5, Nipro, Japan). Six-milliliter fractions of effluents from the arteries perfused with the Krebs-Henseleit solution containing 0.2 mM isobutyl methyl xanthine (IBMX) with or without 100 μ M L-NMMA were collected from 5 to 7 min after initiating the infusion of stimulator, and the levels of cyclic GMP in the effluents were estimated by radioimmunoassay

as described previously (17). The measured values of cyclic GMP were corrected for the body weight (BW), and the levels of cyclic GMP were expressed as fmol cyclic GMP/min/g BW.

L-NMMA was synthesized as described previously (18). ACh, SNP, L-arginine and IBMX were obtained from Sigma Chemical, USA. ANP (human 1-28) was obtained from the Peptide Institute, Osaka, Japan.

Statistical analysis was performed by Student's *t*-test for paired data and one way analysis of variance for unpaired data. The results were expressed as the mean $\pm$ S.E.

RESULTS

Figure 1 shows the time course of changes in levels of cyclic GMP derived from rat mesenteric arteries after infusions of 10⁻⁵ M ACh, 10⁻⁵ M SNP and 10⁻⁷ M ANP. The levels of cyclic GMP were already significantly (*P* < 0.05) elevated at 4 min after beginning the infusions of ACh, SNP and ANP, respectively.

Fig. 1. Time course of changes in cyclic GMP levels in perfused rat mesenteric arteries before and after infusion of 10⁻⁵ M acetylcholine (ACh), 10⁻⁵ M sodium nitroprusside (SNP) and 10⁻⁷ M atrial natriuretic peptide (ANP). Vertical bars indicate the mean $\pm$ S.E. The *P* values (*: *P* < 0.05, **: *P* < 0.01) refer to comparisons with the values before the infusions. ○: ACh (*n* = 4), ●: SNP (*n* = 4), △: ANP (*n* = 4).

The effect of 100 μ M L-NMMA on the basal levels of cyclic GMP is shown in Table 1. L-NMMA reduced the basal levels of cyclic GMP significantly ($P < 0.01$). The addition of 300 μ M L-arginine to L-NMMA restored the basal levels of cyclic GMP.

The effect of 100 μ M L-NMMA on the ACh-induced cyclic GMP formation in rat mesenteric arteries is shown in Fig. 2. Increments in the levels of cyclic GMP after infusion of ACh were significantly ($P < 0.05$) elevated at 10^{-5} M. L-NMMA abolished the elevations of cyclic GMP after infusions of ACh. The addition of 300 μ M L-arginine to L-NMMA restored the elevation of cyclic GMP levels in response to ACh infusion.

The effect of L-NMMA on the SNP-induced cyclic GMP formation in rat mesenteric arteries is shown in Fig. 3. Increments in the levels of cyclic GMP after infusions of SNP were significantly ($P < 0.05$) elevated in the absence as well as the presence of L-NMMA. The increment in the cyclic GMP level after infusion of SNP in the absence of L-NMMA was not significantly different from that in the presence of L-NMMA.

The effect of L-NMMA on the ANP-induced cyclic GMP formation in rat mesenteric arteries is shown in Fig. 4. Increments in the cyclic GMP levels after infusion of ANP were significantly ($P < 0.05$) elevated in the absence as well as the presence of L-NMMA, with no significance differences between the elevated levels.

Table 1. Effect of 100 μ M L-N^G-monomethyl arginine (L-NMMA) and concomitant infusion of 300 μ M L-arginine on basal levels of cyclic GMP in perfused rat mesenteric arteries

Agent	n	Cyclic GMP (fmol/min/g BW)
No addition	5	7.9 $\pm$ 1.0
L-NMMA	6	3.9 $\pm$ 0.1*
L-NMMA + L-arginine	6	7.8 $\pm$ 0.8

Values are the mean $\pm$ S.E. *: $P < 0.01$ versus values for no addition.

Fig. 2. Increments in acetylcholine (ACh)-stimulated cyclic GMP levels in perfused rat mesenteric arteries in the absence or presence of 100 μ M L-N^G-monomethyl arginine (L-NMMA) without or with 300 μ M L-arginine. Vertical bars indicate the mean $\pm$ S.E. The P values (*: $P < 0.05$, **: $P < 0.01$) refer to comparisons with the baselines, and the P values (*: $P < 0.05$, **: $P < 0.01$) refer to comparisons between the indicated pair of columns. □: No addition (n = 5), ▨: L-NMMA (n = 6), ■: L-NMMA + L-arginine (n = 6).

Fig. 3. Increments in sodium nitroprusside (SNP)-stimulated cyclic GMP levels in perfused rat mesenteric arteries in the absence or presence of 100 μ M L-N^G-monomethyl arginine (L-NMMA). Vertical bars indicate the mean $\pm$ S.E. The P values (*: $P < 0.05$, **: $P < 0.01$) refer to comparisons with the baselines. □: No addition (n = 6), ▨: L-NMMA (n = 6).

Fig. 4. Increments in atrial natriuretic peptide (ANP)-stimulated cyclic GMP levels in perfused rat mesenteric arteries in the absence or presence of 100 μ M L-N^G-monomethyl arginine (L-NMMA). Vertical bars indicate the mean $\pm$ S.E. The P values (*: $P < 0.05$, **: $P < 0.01$) refer to comparisons with the baselines. □: No addition ($n = 6$), ▨: L-NMMA ($n = 6$).

DISCUSSION

Formation of NO has been shown to occur in macrophage cell lines (11) and endothelial cells (12, 13). As the biological precursor of NO, L-arginine (12–14) and its derivatives (19) have been found to yield NO. Palmer et al. (12) demonstrated that NO can be synthesized from L-arginine by porcine aortic endothelial cells in culture. The release of NO by bradykinin and Ca^{2+} ionophore was enhanced by infusion of NO from the terminal guanido nitrogen atom(s) of L-arginine. The formation of NO from L-arginine was sensitive to inhibition by arginine analogs including L-NMMA, and the inhibition could be overcome by L-arginine (20). Similarly, generation of NO from endothelial cells (15) and the

EDRF-dependent relaxation in vascular rings (14) are also inhibited by L-NMMA and reversed by L-arginine.

It is considered that the EDRF-induced cyclic GMP formation is also regulated by L-arginine. However, there have been few reports about the effects of L-NMMA on the cyclic GMP formation in arteries. We therefore investigated the effects of L-NMMA on basal and other types of agonist-induced cyclic GMP generation to evaluate the contribution of L-arginine to the cyclic GMP formation in arteries.

Stasch et al. (21) recently reported that cyclic GMP extrusion from perfused aortic rings was not increased in parallel with the increase in cyclic GMP accumulation in the tissue after stimulation by SNP. In the present experiment, we confirmed the time course of changes in cyclic GMP extrusion into the perfusion solution from the mesenteric arteries and observed a significant elevation of the cyclic GMP extrusion after the infusion of agonists. We therefore measured basal and stimulated levels of cyclic GMP in the effluents from the perfused mesenteric arteries as the vascular cyclic GMP formation.

In the present experiments, basal and ACh-induced cyclic GMP formations in the rat mesenteric arteries were markedly inhibited by L-NMMA. Concerning the basal cyclic GMP formation in arteries, the mechanism underlying the basal release of EDRF appears to be different from that for stimulated release in general: in rabbit aortic strips, the basal release of EDRF is unaffected by inhibitors of mitochondrial ATP generation, whereas the stimulated release of EDRF is inhibited (22). It has been found that phosphodiesterase inhibitors do not stimulate EDRF release itself but amplify the effect of the basal EDRF activity (23). The levels of cyclic GMP were measured in the effluents of a perfusion solution containing IBMX, a phosphodiesterase inhibitor, from the rat mesenteric arteries, indicating the basal levels of cyclic GMP well-reflect the basal activity of EDRF in the present experiments. Gold et al. (24) demonstrated re-

cently that L-NMMA caused endothelium-dependent decreases in the basal levels of cyclic GMP that were characteristic of those in endothelium-denuded vessels, and L-NMMA inhibited endothelium-dependent relaxant responses and cyclic GMP formation stimulated by ACh and bradykinin in bovine intrapulmonary artery and vein. These data are consistent with our results. Moreover, the concomitant administration of excess L-arginine restored L-NMMA-induced inhibitions of basal as well as ACh-stimulated cyclic GMP formations in the present experiments. Particularly, concomitant addition of L-arginine with L-NMMA resulted in basal cyclic GMP levels similar to that in the absence of L-NMMA. Based on these findings, it can be considered that L-arginine is necessary not only for the stimulated formation but also the basal formation of cyclic GMP in the artery, and the basal cyclic GMP formation is mainly maintained by the spontaneous release of EDRF.

We investigated the effects of L-NMMA on SNP- and ANP-induced cyclic GMP formations in rat mesenteric arteries, since the cyclic GMP formations induced by these agents are not dependent on EDRF. Relaxation of vascular smooth muscle and the cyclic GMP formation caused by SNP are thought to be dependent on the generation of nitric oxide-free radicals and direct activation of the soluble guanylate cyclase of vascular smooth muscle (3). In contrast, ANP-induced vascular smooth muscle relaxation and cyclic GMP formation do not involve the endothelium (25, 26) and are accompanied by an activation of the particulate guanylate cyclase of vascular smooth muscle (5). In the present experiments, the increment in cyclic GMP levels after infusions of SNP or ANP were the same in the absence and presence of L-NMMA, suggesting that SNP and ANP-stimulated cyclic GMP formations were independent of L-arginine. Recently, Ishii et al. (27) also investigated the effects of L-NMMA on the cyclic GMP formations with SNP and ANP in rat lung fibroblast cells and reported that L-NMMA did not affect the

cyclic GMP formation by SNP and ANP. However, they reported that L-NMMA had no significant effect on the basal cyclic GMP level in rat lung fibroblast cells. This finding conflicts with the results of the present experiments. The difference may be attributable to the use of different cell lines and experimental procedures; in this study, we measured the cyclic GMP levels in the effluents of the perfusion solution containing IBMX that activates the basal activity of EDRF, whereas they did not employ IBMX. However, in both cases, it is indeed suggested that SNP- and ANP-stimulated formation of cyclic GMP in the arteries are independent of L-arginine.

In conclusion, L-NMMA inhibited basal as well as ACh-induced cyclic GMP formations in the rat mesenteric arteries, and excess L-arginine reversed the inhibition of cyclic GMP formation. L-NMMA did not affect the increments in SNP- and ANP-stimulated cyclic GMP levels. From these findings, it is considered that both ACh-stimulated cyclic GMP formation and basal cyclic GMP formation in the mesenteric arteries are maintained by L-arginine as a precursor of EDRF.

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