Effects of Indomethacin, Endothelium-Denudation, Methylene Blue and L-N^G-Monomethyl Arginine on the Vasoactive Effects of Endothelin-3

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ABSTRACT — To clarify the mechanisms underlying the vasoactive effects of endothelin-3 (ET-3), we examined the effects of indomethacin, endothelium-denudation, methylene blue and L-N^G-monomethyl arginine (L-NMMA) on the perfusion pressures of isolated rat mesenteric arteries infused with ET-3. ET-3 at 10^-15 - 10^-8 M elicited significant vasodilations in a dose-related manner, in which 10^-9 and 10^-8 M ET-3 caused biphasic pressure changes involving a transient dilation and subsequent vasoconstriction. Five micromolar indomethacin did not affect the vasodilations and vasoconstrictions induced by ET-3. In endothelium-denuded arteries, 10^-13 - 10^-8 M ET-3 elicited significant vasoconstriction in a dose-related manner without any vasodilation. In the presence of 30 µM methylene blue, the vasodilations induced by ET-3 disappeared. In the presence of 100 µM L-NMMA, 10^-15 - 10^-8 M ET-3 elicited significant vasoconstriction in a dose-related manner without any vasodilation.

Endothelin (ET) was recently isolated from porcine aortic endothelial cells and was reported to be a potent vasoconstrictor (1). Yanagisawa et al. (1) found that ET constricted rat aortic strips in a dose-dependent manner, where the initial dose for constriction was 10^-8 M and the EC_{50} was 5 - 6 x 10^-8 M. However, it has been reported that intravenous bolus injection of ET (0.1 to 3 nM/kg) causes a transient, dose-related depressor response (2, 3). Even in in vitro experiments, vasodilating effects of ET, particularly at low doses, have been demonstrated (4-6).

ET can be distinguished into three different amino acid residues called ET-1, ET-2 and ET-3. It has been reported that different abilities for exerting vasoactive effects exist within this ET family: ET-3 has more potent vasodilating effects compared to ET-1 and ET-2, whereas ET-3 displays lesser vasoconstricting effects (7). We have also found that low doses of ET-3 elicit continuous vasodilations, whereas high
doses (above $10^{-9}$ M) of ET-3 induce a transient vasodilation and subsequent increase in pressure in rat mesenteric arteries (6). It seems likely therefore that low doses of ET-3 have only vasodilating effects, whereas high doses of ET-3 exert vasodilating as well as vasoconstricting effects.

There are few reports concerning the mechanism underlying the different vasoactive effects of low and high doses of ET-3. In an attempt to evaluate the vasoactive effects of ET-3, we examined the effects of indomethacin as an inhibitor of prostaglandin synthesis, endothelium-denudation, methylene blue as a guanylate cyclase inhibitor, and L-N^G^-monomethyl arginine (L-NMMA) as an inhibitor of the synthesis of endothelium-derived nitric oxide (NO) on the perfusion pressures of rat mesenteric arteries infused with ET-3.

**MATERIALS AND METHODS**

Male Wistar rats weighing 250 - 300 g were used. Preparation of isolated rat mesenteric arteries was performed as described previously (8). The rats were anesthetized with pentobarbital, i.p. The superior mesenteric arteries were separated close to the intestine and were perfused at 3 ml/min using an infusion pump (MP-3, Tokyo Rikakikai, Japan) with Krebs-Henseleit solution gassed with 5% CO2 in O2. The perfusion pressure was recorded via the side arm of an arterial cannula employing a pressure transducer (TP-200T, Nihon Kohden, Tokyo). The mesenteric arteries were preperfused for over 60 min and were preconstricted with 100 μM norepinephrine (NE). ET-3 was infused at 1800 μl/hr using a micro infusion pump (SP-5, Nipro, Tokyo).

Indomethacin or methylene blue was dissolved in the perfusion solution. L-NMMA was infused into the mesenteric arteries at 1800 μl/hr using the micro infusion pump. To investigate the role of the endothelium in the vascular response to ET-3, the endothelium was denuded by infusion of Krebs-Henseleit solution containing 0.3% 3-(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS) for 3 min as described previously by Tesfamariam and Halpen (9). Before the experiments, we confirmed the occurrence of endothelium-denudation of the mesenteric arteries treated with CHAPS based on the absence of any acetylcholine-induced vasodilation of the arteries preconstricted with 100 μM NE.

ET-3 was obtained from the Peptide Institute, Osaka, Japan. Indomethacin, CHAPS and methylene blue were obtained from Sigma Chemical, U.S.A. L-NMMA was synthesized as described previously (10).

Statistical analyses were performed by two way analysis of variance for paired and unpaired data. The results were expressed as the mean ± S.E.

**RESULTS**

Typical pressor responses of mesenteric arteries preconstricted with 100 μM NE to $10^{-13}$, $10^{-9}$ and $10^{-8}$ M ET-3 are shown in Fig. 1. Infusion of $10^{-13}$ M ET-3 elicited a continuous vasodilation until wash out, whereas the vasodilations with $10^{-9}$ and $10^{-8}$ M ET-3 were transient, and subsequently, the perfusion pressure with $10^{-8}$ M ET-3 became gradually elevated.

The preconstriction levels of the mesenteric arterial pressures by 100 μM NE with and without several agents are summarized in Table 1. The preconstriction levels of the arteries by 100 μM NE with 30 μM methylene blue and 100 μM L-NMMA were significantly (P < 0.01) higher than that in the case of no addition.

The effects of 5 μM indomethacin, 0.3% CHAPS, 30 μM methylene blue and 100 μM L-NMMA on the vasodilations and vasoconstrictions of perfused mesenteric arteries infused with ET-3 are illustrated in Fig. 2. ET-3 alone elicited dose-related significant (P < 0.01) vasodilations at $10^{-15}$ - $10^{-8}$ M, among which $10^{-9}$ and $10^{-8}$ M ET-3 caused biphasic pressure changes involving transient dilations followed by significant (P < 0.01) constrictions. In the presence of 5 μM indomethacin,
Fig. 1. Representative recordings of typical changes in the perfusion pressure of mesenteric arteries preconstricted with 100 μM norepinephrine after infusions of 10^{-13}, 10^{-9} and 10^{-8} M endothelin (ET)-3. The negative spikes are artifacts caused by transient discontinuance of the ET infusions.

Table 1. Mean perfusion pressures of rat mesenteric arteries preconstricted with 100 μM norepinephrine and treated or untreated with several agents

| Agent                  | n  | Pressure (mmHg) |
|------------------------|----|----------------|
| No addition            | 11 | 46.6 ± 4.2     |
| 5 μM indomethacin      | 12 | 40.7 ± 5.3     |
| 0.3% CHAPS             | 7  | 37.1 ± 3.4     |
| 30 μM methylene blue   | 10 | 132.8 ± 10.8   |
| 100 μM L-NMMA          | 7  | 73.7 ± 7.3     |

Values are the mean ± S.E. CHAPS: 3-[3-Cholamidopropyl]-dimethyl-ammonio]-1-propane sulfonate. L-NMMA: L-N^4'-monomethyl arginine. *: P < 0.01 versus values for no addition.

10^{-13} - 10^{-8} M ET-3 elicited significant (P < 0.01) vasodilations in a dose-related manner, and 10^{-8} M ET-3 subsequently caused a significant (P < 0.05) vasoconstriction as part of a biphasic pressure change. In the mesenteric arteries endothelium-denuded with 0.3% CHAPS, ET-3 did not cause any vasodilation, whereas 10^{-13} - 10^{-8} M ET-3 elicited significant (P < 0.05) vasoconstrictions in a dose-related manner. In the presence of 30 μM methylene blue, ET-3 did not induce any significant vasodilation, whereas 10^{-13} (P < 0.05) and 10^{-8} (P < 0.01) M ET-3 caused significant vasoconstrictions. In the presence of 100 μM L-NMMA, ET-3 elicited a significant (P < 0.05) vasodilation only at 10^{-8} M, whereas 10^{-15} - 10^{-8} M ET-3 caused significant (P < 0.05) vasoconstrictions in a dose-related manner (Fig. 2).

Comparisons of the degrees of vasodilation and vasoconstriction induced by 10^{-8} M ET-3 in mesenteric arteries preconstricted with 100 μM NE, and treated with several agents, are presented in Table 2. There were no significant differences in the degrees of vasodilation...
Table 2. Comparisons of the degrees of vasodilation and vasoconstriction induced by $10^{-8}$ M endothelin-3 in mesenteric arteries preconstricted with 100 $\mu$M nor-epinephrine and treated or untreated with several agents

| Agent                  | n  | Vasodilation | Vasoconstriction |
|------------------------|----|--------------|-----------------|
| No addition            | 11 | 21.6 ± 2.9   | 19.9 ± 2.3      |
| 5 $\mu$M indomethacin  | 12 | 17.5 ± 2.6   | 11.7 ± 4.3      |
| 0.3% CHAPS             | 7  | 0 ± 0**      | 42.7 ± 9.6*     |
| 30 $\mu$M methylene blue | 10 | 1.7 ± 0.8**  | 12.2 ± 2.5*     |
| 100 $\mu$M L-NMMA      | 7  | 5.6 ± 2.2**  | 29.7 ± 4.8      |

Values are the mean ± S.E. CHAPS: 3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate, L-NMMA: L-N$^{+}$-monomethyl arginine. *: $P < 0.05$, **: $P < 0.01$ versus values for no addition.

DISCUSSION

In the present experiments, $10^{-15} - 10^{-8}$ M ET-3 elicited vasodilations in a dose-related manner in rat mesenteric arteries. In particular, low doses, $10^{-15} - 10^{-11}$ M, of ET-3 elicited continuous vasodilations without any vasoconstriction, whereas $10^{-9}$ and $10^{-8}$ M ET-3 elicited transient vasodilations and subsequent vasoconstrictions in a biphasic pattern.

The mechanism of the vasodilating effects of ET-3 has been reported to involve contributions of endothelium-derived relaxing factor (EDRF) (4, 6, 11) or prostaglandins (12). Warner et al. (4) observed that vasodilations in isolated perfused mesenteric arteries of the rat in response to 1 – 300 pM ET-3 were inhibited by removal of the endothelium, methylene blue, or hemoglobin, suggesting that the vasodilations were due to the release of EDRF. We have found that the vasodilating effects of low doses of ET-3 on mesenteric arteries were dependent on the extracellular Ca$^{2+}$ and the endothelium, and they were accompanied by elevated levels of cyclic GMP derived from the arteries, also suggesting the possibility of a contribution of EDRF (6).

Since the degrees of vasodilation by ET-3 were not reduced by indomethacin in the present experiments, it can be considered that the vasodilating effects of ET-3 are not associated with prostaglandin production.

In the present experiments, ET-3 induced dose-dependent vasoconstrictions of the endothelium-denuded arteries even at $10^{-13}$ and $10^{-11}$ M, which elicits continuous vasodilations of intact arteries. These data suggest that the vasodilating effects of ET-3 depend on the endothelium, and that the vasodilating effects possibly overcame the vasoconstricting effects of ET-3 at the $10^{-13}$ and $10^{-11}$ M levels. Even low doses of ET-3 may therefore have vasodilating effects through the endothelium as well as direct vasoconstricting effects on the vascular smooth muscle. It is speculated that different ET-3 receptors may exist on the endothelium and vascular smooth muscle, of which the former displays a high affinity and the latter have a low affinity. Low doses of ET-3 may
act on the endothelium to cause vasodilation, whereas high doses of ET-3 may act on both receptors to provoke biphasic pressure changes.

In the present experiments, the vasodilating effects of ET-3 were inhibited by methylene blue, indicating that such vasodilating effects are associated with EDRF. One EDRF has been identified chemically as nitric oxide (NO) (13). It has been reported that endothelial cells in culture synthesized NO from the terminal guanido nitrogen atom(s) of the amino acid L-arginine (14, 15). It has recently been found that the synthesis of NO and the vasodilating effects of EDRF are inhibited by L-NMMA, an analog of L-arginine (16, 17).

We demonstrated recently that L-NMMA abolished the vasodilation induced by $10^{-13}$ M ET-3 in rat mesenteric arteries, and that L-arginine, not D-arginine, restored the vasodilation, suggesting that the vasodilating effects of $10^{-13}$ M ET-3 are associated with endothelium-derived NO (18). We therefore examined the effects of L-NMMA on the vasodilating effects of low and high doses of ET-3 in the present experiments, and found that L-NMMA abolished the vasodilations induced by low doses, below $10^{-9}$ M, of ET-3 and that $10^{-15} - 10^{-8}$ M ET-3 elicited vasoconstrictions in a dose-related manner in the presence of L-NMMA. These pressure effects of ET-3 with L-NMMA are similar to those of ET-3 on arteries endothelium-denuded by CHAPS. It is possible that the vasoconstricting effects of ET-3 on the vascular smooth muscle might be uncovered through the action of L-NMMA which inhibited the release of endothelium-derived NO by ET-3.

Vasodilation by $10^{-8}$ M ET-3 was observed, but at a significantly reduced level, in the presence of 100 µM L-NMMA in the present experiments. It is possible that the effect of 100 µM L-NMMA might be still incomplete in inhibiting EDRF release by $10^{-9}$ M ET-3, since a dose-dependency of EDRF inhibition by L-NMMA has been reported (19). It has been found that ET-3 can stimulate the release of prostacyclin (20, 21). Therefore, the remaining vasodilation by $10^{-8}$ M ET-3 in the presence of L-NMMA may indicate the possibility that $10^{-8}$ M ET-3 may have stimulated prostacyclin release. However, there were no differences in the degrees of vasodilation by $10^{-8}$ M ET-3 between the cases with and without indomethacin in the present experiments, indicating that it is unlikely that prostaglandins contribute to the vasodilating effects of $10^{-8}$ M ET-3.

L-NMMA, an analog of L-arginine, thus inhibited the vasodilations induced by low and high doses of ET-3 and caused vasoconstrictions even at low doses of ET-3, indicating that the vasodilating effects of low doses of ET-3 through the endothelium overcome the vasoconstricting effects, and that the vasodilating effects of ET-3 are associated with EDRF as an endothelium-derived NO.

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