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Platelets enhance contractility in perfused rat mesenteric arteries: Involvement of endothelin-1

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Abstract

We investigated the effects of platelet supernatant on pressor responses to norepinephrine in isolated perfused rat mesenteric arteries. Perfusion of the arteries with platelet supernatant for 2 h markedly enhanced the pressor responses to norepinephrine (10^{-6} and 3×10^{-6} M). This enhancement was significantly inhibited by phosphoramidon (10^{-4} M), an endothelin converting enzyme inhibitor. Both BQ788 [*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine] (10^{-6} M), an endothelin ET_B receptor antagonist, and bosentan (Ro47-0203, 4-tert-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2́-bipyrimidin-4-yl]-benzenesulfonamide) (10^{-5} M), a nonselective endothelin receptor antagonist, also prevented the potentiation of responses to norepinephrine evoked by platelet supernatant, but FR139317 ((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methyl-pentanoyl] amino-3-[3-(1-methyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl) propionic acid) (10^{-6} M), an endothelin ET_A receptor antagonist, had little effect. Suppressor doses of endothelin-1 (3×10^{-10} M) or sarafotoxin S6c (S6c) (3×10^{-10} M) potentiated significantly the norepinephrine-induced vasoconstriction, in the same preparation. Moreover, supernatant-induced enhancement of pressor responses to norepinephrine was markedly suppressed by TGF-β1 neutralizing antibody. Transforming growth factor-β1 (TGF-β1) (40 pM) also significantly enhanced the pressor responses to norepinephrine (10^{-6} M) and this enhancement was significantly inhibited by phosphoramidon. These results suggest that platelet-derived TGF-β1 stimulates the vascular production of endothelin-1 and thereby enhances vasoconstrictor responses to norepinephrine. Platelet-induced enhancement of vasoconstrictor responses to norepinephrine seems to be mainly mediated by endothelin ET_B receptor, in rat mesenteric arteries. © 1997 Elsevier Science B.V.

Keywords: Endothelin-1; Endothelin ET_B receptor; Sarafotoxin S6c; Norepinephrine; Mesenteric artery; TGF-β1 (transforming growth factor-β1)

1. Introduction

Endothelin-1, a highly potent vasoconstrictor peptide with 21 amino acid residues, was identified in the culture supernatant of porcine aortic endothelial cells (Yanagisawa et al., 1988). Its wide variety of actions have been studied and this peptide was considered to participate in pathological states such as cerebral vasospasm after subarachnoid hemorrhage (Matsumura et al., 1991a), pulmonary hypertension (Stewart et al., 1991) and myocardial infarction (Watanabe et al., 1991). Angiotensin II, thrombin, arginine–vasopressin and transforming growth factor-β1 (TGF-β1) have been reported to regulate endothelin-1 gene expression at the transcriptional level (Yanagisawa et al., 1988; Kurihara et al., 1989; Imai et al., 1992; Tomobe et al., 1993). These agents also enhance endothelin-1 secretion from endothelial cells.

In endothelial cells, endothelin-1 is synthesized from a 203-amino acid prepro endothelin-1 cleaved by a dibasic pair-specific endopeptidase to a 39 amino acid peptide big endothelin-1 (Yanagisawa et al., 1988). Big endothelin-1 is then converted to mature endothelin-1 by an endopeptidase, termed endothelin converting enzyme. The most relevant candidate for physiological endothelin converting enzyme is membrane-bound neutral metalloprotease, which is phosphoramidon-sensitive (Matsumura et al., 1990a,b, 1991b; Pollock and Opgenorth, 1991). It has been found that phosphoramidon inhibits the pressor effect of big endothelin-1, without affecting that of endothelin-1 (Matsumura et al., 1990a; Pollock and Opgenorth, 1991).

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Ohlstein et al. (1991) demonstrated that platelets augment endothelin-1 secretion from cultured endothelial cells by stimulating the expression of prepro endothelin-1 mRNA. Because platelets can release TGF-β1 during degranulation, TGF-β1 may be a potential mediator of platelet-induced stimulation of endothelin-1 production. More recent studies from our laboratory indicated that platelets stimulate endothelial endothelin-1 production mainly through the release of TGF-β1, since the effect of platelets was significantly suppressed by treatment with TGF-β1-neutralizing antibody (Matsumura et al., 1994). Such a phenomenon might have pathological and/or physiological importance, especially if it is involved in vascular diseases. Although platelets-induced stimulation of endothelin-1 production has been well studied, little is known of the pathological or physiological role of this phenomenon.

In the present study, we observed that platelet supernatant enhances pressor responses to norepinephrine in perfused rat mesenteric arteries. We asked whether endothelin-1 contributes to the enhancement induced by platelet supernatant, using phosphoramidon and endothelin receptor antagonists, FR139317, bosentan and BQ788. To date, several endothelin receptor antagonists have been used to characterize the physiological roles of endothelin-1 and its receptor subtypes. Among them, FR139317 is a selective endothelin ET_A receptor antagonist which inhibits endothelin-1-induced vasoconstrictor effects, in vitro and in vivo (Sogabe et al., 1993). Bosentan is a nonselective endothelin receptor antagonist which competitively inhibits contractions induced by endothelin-1 in the isolated rat aorta (ET_A) and by the selective endothelin ET_B agonist sarafotoxin S6c (S6c) in the rat trachea (Clozel et al., 1994). BQ788 is a selective endothelin ET_B receptor antagonist which competitively antagonizes the vasoconstriction induced by a selective endothelin ET_B agonist BQ3020 (Ishikawa et al., 1994). We report here that platelet supernatant enhances pressor responses to norepinephrine in perfused rat mesenteric arteries and that this enhancement is apparently mediated by the action of endothelin-1, mainly via the endothelin ET_B receptor.

2. Materials and methods

2.1. Isolated perfused rat mesenteric artery

Experiments were performed using male Sprague–Dawley rats, weighing 300–350 g. The animals were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and the abdomen was opened by a midline incision. The superior mesenteric artery was cannulated with a polyethylene catheter and perfused at a constant flow rate of 3 ml/min with Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The perfusate was constantly bubbled with 95% O_2–5% CO_2, to adjust the pH at 7.4–7.6 and for oxygenation. The mesentery was placed in a siliconized 30 ml organ bath maintained at 37–38°C and perfused in the open system for 20 min to avoid contamination by plasma components, thereafter the perfusion system was changed to the closed system. Changes in perfusion pressure were measured at a point close to the mesentery by means of a pressure transducer (AP 601G, Nihonkohden, Osaka) and recorded on a polygraph (RM 6000G, Nihonkohden, Osaka).

2.2. Preparation of platelet supernatant

Rat platelet-rich plasma was centrifuged at $750 \times g$ for 15 min and the pellet was suspended in 12 mmol/l Tris–HCl buffer containing 139 mmol/l NaCl (pH 7.4). The suspension was centrifuged at $600 \times g$ for 10 min and the pellet finally resuspended in DMEM, with care taken to prevent aggregation. The preparations resulted in a cell population of $> 98\%$ platelets. To obtain the supernatant of platelets, the final suspension in DMEM was adjusted to 6×10^8 cells/ml and the preparation was incubated at 37°C for 4 h with thrombin (0.1 U/ml), in a CO_2 incubator. After the incubation, the suspension was centrifuged at $1700 \times g$ for 10 min and the resulting supernatant was used for experiments.

2.3. Experimental protocols

Following an equilibration period of 20 min, pressor responses to three concentrations of norepinephrine (10^{-6}, 3×10^{-6} and 10^{-5} M) was obtained as basal control responses. After this control experiments, platelet supernatant was perfused for 2 h and then the same experiments were repeated. In some experiments, phosphoramidon (10^{-4} M), FR139317 (10^{-6} M), bosentan (10^{-5} M) and BQ788 (10^{-6} M) were treated 15 min prior to the start of perfusion with platelet supernatant. To study the effects of TGF-β1 neutralizing antibody on platelet supernatant-induced action, platelet supernatant was preincubated with the antibody (10 μg/ml) for 2 h at 37°C and then the arteries were perfused with this preparation for 2 h. The appropriate time control experiments were carried out in the absence of platelet supernatant.

To study the effects of synthetic endothelin-1 and S6c on norepinephrine-induced contractions, the arteries were perfused with a suppressor dose of endothelin-1 (3×10^{-10} M) or S6c (3×10^{-10} M) for 15 min. Cumulative response to norepinephrine were obtained before and after perfusion with endothelin-1 or S6c.

In a different series of experiments, to examine the effects of TGF-β1 on pressor responses to norepinephrine, the arteries were perfused with TGF-β1 for 2 h.

2.4. Drugs

Endothelin-1, S6c and phosphoramidon were purchased from Peptide Institute (Osaka). Endothelin-1 and S6c were dissolved in saline solution containing 0.1% heat-in-

activated bovine serum albumin, and phosphoramidon was dissolved in saline. Human TGF-β1 and its antibody were obtained from R & D Systems (Minneapolis, MN). FR139317 ((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methylpentanoyl] amino-3-[3-(1-methyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl) propionic acid), a kind gift from Fujisawa Pharmaceutical, Osaka, was dissolved in 1 N NaOH and then diluted with saline. BQ788 [*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine], a kind gift from Banyu Pharmaceutical, Tsukuba, was dissolved in dimethyl sulfoxide and then diluted with saline. Bosentan (Ro47-0203, 4-tert-butyl-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2́-bipyrimidin-4-yl]-benzene-sulfonamide), a kind gift from Hoffmann–La Roche, Basel, was dissolved in dimethyl sulfoxide and then diluted with saline. Other chemicals were purchased from Wako Pure Chemical Industries (Osaka).

2.5. Statistical analysis

All values were expressed as mean ± S.E.M. For statistical analysis, we used the paired Student's *t*-test for two-sample comparisons and one-way analysis of variance combined with Duncan's new multiple range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Contractile responses of perfused rat mesenteric arteries to norepinephrine

As shown in Table 1, norepinephrine (10^{-6}–10^{-5} M) elicited a concentration-related contraction in perfused rat mesenteric arteries. Pressor responses to norepinephrine (3×10^{-6} and 10^{-5} M) were significantly increased compared with the basal control responses (135.7 ± 37.7 and 203.6 ± 57.7% increase, respectively).

Table 1
Pressor responses (in mm Hg) to norepinephrine in perfused rat mesenteric arteries

	NE (M)		
	10^{-6}	3×10^{-6}	10^{-5}
First (basal)	3.1 ± 0.9	8.0 ± 1.5	31.3 ± 9.1
Second	4.3 ± 1.1	18.1 ± 3.2[a]	81.0 ± 12.3[a]

NE (10^{-6}–10^{-5} M) were cumulatively added to perfusate as basal control experiment (first). After the first experiment, the arteries were perfused for 2 h and then the same dose–response experiments were repeated (second). Each value represents the mean ± S.E.M. from 5 separate experiments. Time-dependent changes of perfusion pressure were examined by Student's paired *t*-test.
[a] $p < 0.01$ compared with the basal control value (first).

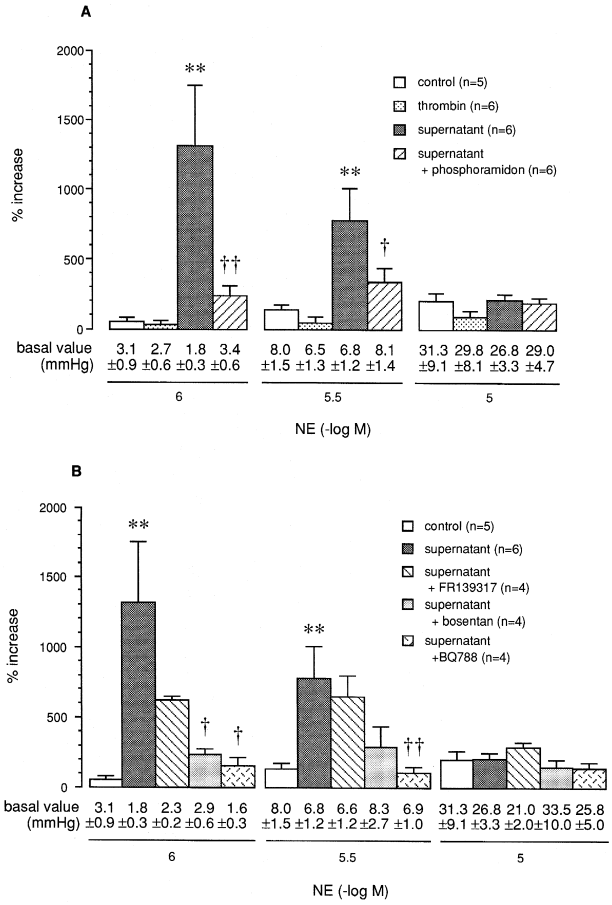

Fig. 1. Effects of platelet supernatant on pressor responses to norepinephrine in perfused rat mesenteric arteries. The arteries were perfused with platelet supernatant for 2 h with or without phosphoramidon (A) or endothelin receptor antagonists (B) pretreatment. Responses are expressed as percent increase of basal control responses before perfusion with platelet supernatant. The basal control responses of each experimental group are shown below the columns. Columns and bars represent the mean ± S.E.M. $^{**}P < 0.01$, compared with the value in the time control experiment. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with the value observed with platelet supernatant.

3.2. Effects of platelet supernatant on norepinephrine-induced contractions

Fig. 1 shows the basal control responses to norepinephrine before various treatments and the change of contractility to norepinephrine after each treatment. When the arteries were perfused with platelet supernatant for 2 h, the contractile responses to norepinephrine at concentrations of 10^{-6} and 3×10^{-6} M were markedly potentiated (1311.2 ± 438.5 and 779.9 ± 223.8% increase, respectively, compared with the basal control values) and these alterations were statistically significant, compared with those obtained in the time control experiments (51.0 ± 28.1%, 10^{-6} M; 135.7 ± 37.7% increase, 3×10^{-6} M) (Fig. 1A). No significant alterations were observed at 10^{-5} M (210.5 ± 38.7%, platelet supernatant; 203.6 ± 57.7% increase, time control). Thrombin (0.1 U/ml), which was

utilized to obtain the platelet supernatant (see Section 2), did not affect the contractility to norepinephrine. When phosphoramidon (10^{-4} M), an endothelin converting enzyme inhibitor, was pretreated, platelet-induced enhancement of pressor responses to norepinephrine was significantly attenuated. No significant differences were observed in basal control responses of all experimental groups.

3.3. Effects of endothelin receptor antagonists on enhancement of contractile responses to norepinephrine, as induced by platelet supernatant

When bosentan was added to the perfusate 15 min prior to perfusion with platelet supernatant, platelet-induced enhancement of contractile responses to norepinephrine were significantly attenuated (from 1311.2 ± 438.5 to 232.9 ± 45.1% increase for 10^{-6} M and from 779.9 ± 223.8 to 291.7 ± 141.0% increase for 3×10^{-6} M of norepinephrine, respectively) (Fig. 1B). Similar significant suppressive effects were observed using BQ788, but no significant alteration was seen in case of FR139317.

3.4. Effects of endothelin-1 and sarafotoxin S6c on norepinephrine-induced contractions

In time control experiments, the increase in contractility was 31.6 ± 11.1, 21.0 ± 12.1 and 70.6 ± 11.3% for 10^{-6}, 3×10^{-6} and 10^{-5} M of norepinephrine, respectively. When the arteries were perfused with a suppressor dose of endothelin-1 (3×10^{-10} M) for 15 min, there was a significant potentiation of the contraction induced by norepinephrine at a concentration of 10^{-6} M (Fig. 2). The increase in contractility was 232.0 ± 40.0% for 10^{-6} M of norepinephrine. Pressor responses to 3×10^{-6} M of norepinephrine tended to be potentiated by endothelin-1, but this effect was not statistically significant. Similarly, S6c (3×10^{-10} M) potentiated the contractile responses to norepinephrine (201.4 ± 55.8% increase at 10^{-6} M and 233.3 ± 56.5% increase at 3×10^{-6} M of norepinephrine, respectively).

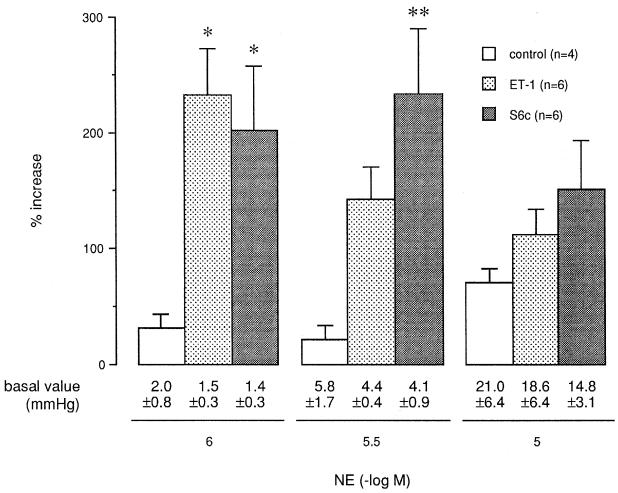

Fig. 2. Effects of endothelin-1 and sarafotoxin S6c (S6c) on pressor responses to norepinephrine in perfused rat mesenteric arteries. The arteries were perfused with endothelin-1 or S6c for 15 min. Responses are expressed as percent increase of basal control responses before the addition of endothelin-1 or S6c. The basal control responses of each experimental group are shown below the columns. Columns and bars represent the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the value in time control experiment.

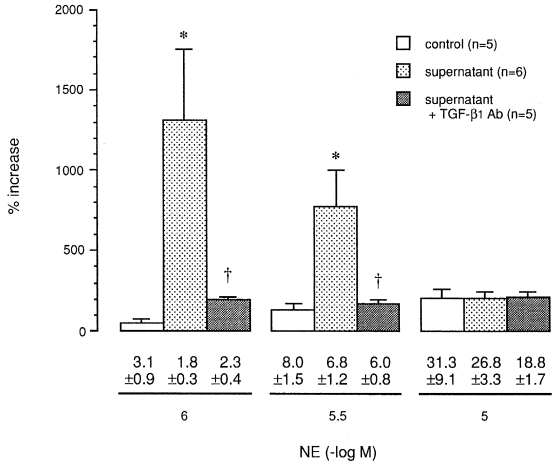

Fig. 3. Effects of TGF-β1 neutralizing antibody on platelet supernatant-induced enhancement of pressor responses to norepinephrine in perfused rat mesenteric arteries. The supernatant was preincubated with the antibody (10 μg/ml) for 2 h at 37°C, and the arteries were perfused with this preparation for 2 h. Responses are expressed as percent increase of basal control responses before perfusion with supernatant or antibody-treated supernatant. The basal control responses of each experimental group are shown below the columns. Columns and bars represent the mean ± S.E.M. $^{**}P < 0.01$, compared with the value in the time control experiment. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with the value observed with platelet supernatant.

3.5. Effects of TGF-β1 neutralizing antibody on enhancement of contractile responses to norepinephrine, as induced by platelet supernatant

When TGF-β1 neutralizing antibody (10 μg/ml) was pretreated to the platelet supernatant, platelet-induced enhancement of contractile responses to norepinephrine were markedly attenuated (200.0 ± 19.2 and 175.5 ± 24.8% increase for 10^{-6} and 3×10^{-6} M of norepinephrine) (Fig. 3).

3.6. Effects of TGF-β1 on norepinephrine-induced contractions

Perfusion of the arteries with TGF-β1 (40 pM) for 2 h significantly potentiated the contraction induced by 10^{-6} M of norepinephrine (Fig. 4). The increase in contractility was 202.3 ± 29.4% for norepinephrine at a concentration of 10^{-6} M (51.0 ± 28.1% increase in time control experi-

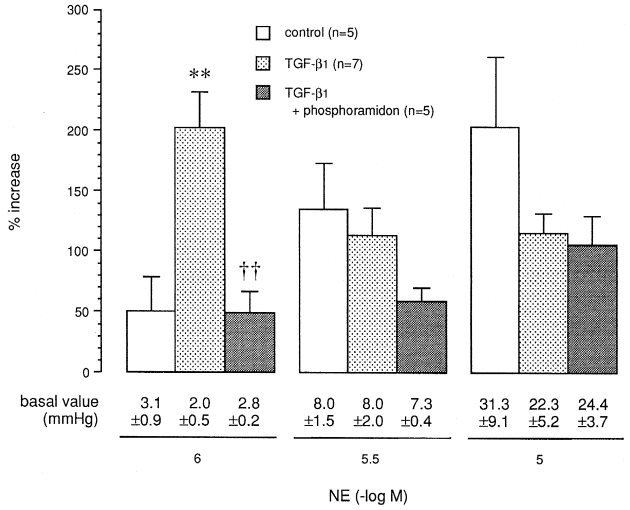

Fig. 4. Effects of transforming growth factor-β1 (TGF-β1) on pressor responses to norepinephrine in perfused rat mesenteric arteries. The arteries were perfused with TGF-β1 for 2 h with or without phosphoramidon pretreatment. Responses are expressed as percent increase of basal control responses before perfusion with TGF-β1. The basal control responses of each experimental group are shown below the columns. Columns and bars represent the mean ± S.E.M. $^{*}P < 0.05$, compared with the value in time control experiment. $^{\dagger}P < 0.05$, compared with the value observed with platelet supernatant.

ment). Pretreatment of the arteries with phosphoramidon significantly inhibited the potentiating effect of TGF-β1 to the basal level.

4. Discussion

The present study demonstrated that perfusion of rat mesenteric arteries with platelet supernatant markedly enhanced the norepinephrine-induced contraction. Although the norepinephrine-induced contraction increased with the time of perfusion (see Table 1, time control experiments), the increase was significantly greater in the presence of platelet supernatant. Thrombin, which was used to obtain the platelet supernatant, by itself had no apparent effect on contractile responses to norepinephrine, which suggests that platelets-derived substance directly or indirectly potentiates the norepinephrine-induced contraction. We used low concentrations of thrombin (0.1 U/ml) which had no significant effects on endothelin-1 production in bovine pulmonary artery endothelial cells, but was effective for producing platelet aggregation (Ohlstein et al., 1991).

Recent studies have demonstrated that platelets stimulate the endothelin-1 release in endothelial cells by stimulating endothelin-1 gene expression (Ohlstein et al., 1991; Mikkola et al., 1993; Umekawa et al., 1993) and that this release is inhibited by phosphoramidon (Umekawa et al., 1994). In the present study, phosphoramidon inhibited the platelet-induced enhancement of contractions induced by norepinephrine in rat mesenteric arteries. Thus, local production of endothelin-1 in these arteries may mediate the potentiation of the contractile responses to norepinephrine, as evoked by platelet supernatant. To gain support for this hypothesis, we determined whether the suppressor dose of exogenous endothelin-1 would potentiate contractions induced by norepinephrine, and found that endothelin-1 potentiates the contractile responses to norepinephrine. Several groups of workers have demonstrated that a subthreshold concentration of endothelin-1 potentiates the vasoconstrictor effects of norepinephrine (Yang et al., 1990; Henrion and Laher, 1993) and serotonin (Yang et al., 1990; Wong-Dusting et al., 1991), in the rabbit aorta and ear artery and human left anterior descending coronary artery. Taken together, it is reasonable to consider that platelet supernatant-induced potentiation of the contractile responses to norepinephrine in rat mesenteric arteries is due to endothelin-1, the production of which is enhanced by the platelet-derived substances.

In this study, we found that both platelet supernatant and endothelin-1 potentiated the contraction induced by lower concentrations of norepinephrine (10^{-6} and 3×10^{-6} M), but not by a high concentration of norepinephrine (10^{-5} M), while the increase in contraction observed in time control experiments was higher when the concentration of norepinephrine was high. This may explain the lack of the potentiating effect on a high dose of norepinephrine, in that the responses to this dose of norepinephrine are submaximal and not potentiated.

Two distinct subtypes of endothelin receptors, ET_A and ET_B, have been characterized and cloned from bovine and rat lung, respectively (Arai et al., 1990; Sakurai et al., 1990). Endothelin ET_A receptors, which occur mainly on vascular smooth muscle cells, mediate vasoconstriction (Lüscher et al., 1993), and endothelin ET_B receptors, which locate predominantly on endothelial cells, mediate vasodilation by generation of endothelium-derived relaxing factor and prostacyclin (Warner et al., 1989; Lüscher et al., 1993). However, it became apparent that non-ET_A receptors mediate some of the vasoconstrictor actions of endothelin-1. The renal vasculature and several other arterial and venous vascular beds appear to carry endothelin ET_B receptor-mediated constrictor elements in vitro (Clozel et al., 1992; Cristol et al., 1993). In the present study, BQ788 and bosentan effectively prevented the potentiation of contractile responses to norepinephrine, as evoked by platelet supernatant. Furthermore, S6c significantly potentiated the contractions induced by norepinephrine, thereby suggesting the involvement of endothelin ET_B receptor-mediated events. However, the possibility that the effects of endothelin-1 through endothelin ET_A receptor might partly contribute to platelet-induced potentiation of contractile responses to norepinephrine would need to be excluded, since the effect of platelet supernatant at a low dose of norepinephrine tended to be attenuated by FR139317.

Platelets release various bioactive substances such as serotonin and TGF-β1. Several studies have demonstrated that TGF-β1 effectively stimulates prepro endothelin-1

mRNA expression and increases the release of endothelin-1 (Kurihara et al., 1989; Wargner et al., 1992). We found that platelets stimulate endothelial endothelin-1 production mainly by releasing TGF-β1 (Matsumura et al., 1994). Therefore, we evaluated the effects of TGF-β1 neutralizing antibody on platelet supernatant-induced actions. As a result, platelet supernatant-induced enhancement of contractile responses to norepinephrine was markedly attenuated by TGF-β1 neutralizing antibody. Furthermore, TGF-β1 significantly potentiated the contractions induced by norepinephrine. Our results clearly indicated that TGF-β1 potentiates significantly the contractility to norepinephrine. Although the concentration of TGF-β1 we used was equivalent to that in the platelet supernatant, the effect of TGF-β1 was considerably less compared with that seen with platelet supernatant. The reason for this discrepancy between platelet supernatant- and TGF-β1-induced actions is unclear. One possibility is that active TGF-β1 concentration in platelet supernatant may be gradually increased by activation of its latent form during the perfusion period.

Taken together, we conclude that platelet-derived TGF-β1 stimulates the local production of endothelin-1 in rat mesenteric arteries and enhances the vasoconstrictor responses to norepinephrine, mainly via endothelin ET_B receptor. Endothelin-1 may have a role to play in vascular diseases associated with platelet activation such as disseminated intravascular coagulation (Asakura et al., 1992) and atherosclerosis (Boulanger et al., 1992), in which interaction between platelets and endothelial cells has a key role (Ware and Heistad, 1993). Further studies are under way to examine the pathophysiological role of interactions between platelets and endothelin-1.

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