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# Possible involvement of endothelium-derived hyperpolarizing factor (EDHF) in the depressor responses to platelet activating factor (PAF) in rats

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**1** In anaesthetized rats, platelet activating factor (PAF; 1 $\mu$g kg$^{-1}$) decreased mean arterial blood pressure by around 60 mmHg ($n=18$). This depressor response was completely blocked by the PAF antagonist, CV-6209 (1 mg kg$^{-1}$), indicating the role of PAF-specific receptor in the response.

**2** N$^{G}$-nitro-L-arginine methyl ester (L-NAME; 50 mg kg$^{-1}$), an NO synthase inhibitor, profoundly elevated systemic blood pressure ($n=19$), indicating an important role of NO in the basal blood pressure regulation. The depressor response to PAF (1 $\mu$g kg$^{-1}$) normalized against that to sodium nitroprusside (SNP) (10 $\mu$g kg$^{-1}$) was not substantially different between rats treated without and with L-NAME ($n=4$). In contrast, the depressor effect of acetylcholine (0.03–1.0 $\mu$g kg$^{-1}$) normalized against that of SNP (10 $\mu$g kg$^{-1}$) was significantly attenuated by L-NAME ($n=5$).

**3** Charybdotoxin (0.4 mg kg$^{-1}$) plus apamin (0.2 mg kg$^{-1}$) significantly attenuated the depressor response to PAF (1 $\mu$g kg$^{-1}$) ($n=5$) without affecting the blood pressure change due to SNP (1 mg kg$^{-1}$) ($n=3$). Charybdotoxin (0.4 mg kg$^{-1}$) ($n=4$) or apamin (0.2 mg kg$^{-1}$) ($n=4$) alone did not affect the PAF-induced depressor response.

**4** These findings suggest that EDHF may make a significant contribution to the depressor response to PAF in rats. Although NO plays the determinant role in the basal blood pressure regulation, its contribution to PAF-produced depressor response seems to be less as compared with that to the depressor response to acetylcholine.

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**Keywords:** PAF (platelet activating factor); nitric oxide (NO); endothelium-derived hyperpolarizing factor (EDHF); charybdotoxin; apamin; systemic arterial blood pressure in rats

**Abbreviations:** EDHF, endothelium-derived hyperpolarizing factor; L-NAME, N$^{G}$-nitro-L-arginine methyl ester; NO, nitric oxide; PAF, platelet activating factor; ACh, acetylcholine; SNP, sodium nitroprusside; $PGI_2$, prostacyclin

## Introduction

Platelet activating factor (PAF) is an endogenous phosphoglyceride derivative (1-*O*-alkyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine) (Hanahan *et al*., 1980), which is produced in stimulated leucocytes, platelets, macrophages and endothelium of various species (Kamata *et al*., 1996b). In addition to its potent effects on platelets, PAF has been described to produce a profound and dose-dependent depressor response in various animal species, e.g., normotensive and spontaneously hypertensive rats, rabbits, guinea-pigs and dogs (Tanaka *et al*., 1983). The depressor effects of PAF have been postulated to be at least partly attributable to the dilatation of resistance vessels (Tanaka *et al*., 1983; Yamanaka *et al*., 1992). Actually, PAF was demonstrated to produce relaxations of the isolated rat aortic (Kasuya *et al*., 1984; Moritoki *et al*., 1992; Shigenobu *et al*., 1987) and mesenteric (Chiba *et al*., 1990; Kamata *et al*., 1996a; 1989) arteries. Other mechanisms responsible for the depressor response to PAF are: pulmonary hypertension (Laurindo *et al*., 1989); decreased blood volume (Bessin *et al*., 1983); reduction of cardiac output (King *et al*., 1995; Yamanaka *et al*., 1992); attenuation of venous return due to venodilatation (Yamanaka *et al*., 1992).

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Relaxation of arterial blood vessels in response to PAF is dependent on the presence of intact endothelium (Chiba *et al*., 1990; Kamata *et al*., 1989; Kasuya *et al*., 1984; Moritoki *et al*., 1992) as in the case of other relaxations produced by various vasoactive substances (Furchgott, 1984). It is now generally recognized that both NO (endothelium-derived relaxing factor: EDRF; Furchgott, 1984; Ignarro *et al*., 1987; Palmer *et al*., 1987) and endothelium-derived hyperpolarizing factor(s) (EDHF(s); Garland *et al*., 1995; Mombouli & Vanhoutte, 1997; Taylor & Weston, 1988) are responsible for endothelium-dependent vascular relaxations. NO is a principal determinant for the regulation of basal blood pressure and is thus physiologically significant in circular systems (Aisaka *et al*., 1989b; Rees *et al*., 1989). By contrast, a physiological role of EDHF has not been fully established when compared to NO. The role of EDHF-activated mechanisms has been postulated to serve either in parallel, or as a secondary 'backup' in the event of a reduction in the NO component and thus, EDHF may have a major role in maintaining 'normal' vascular tone in disease states, in which NO function is lost (Garland *et al*., 1995).

Pharmaco-mechanical evidence obtained with isolated vascular preparations indicates that endothelium-derived NO mainly accounts for the endothelium-dependent relaxa-

tion produced by PAF (Chiba *et al*., 1990; Kamata *et al*., 1996a; Moritoki *et al*., 1992). These findings imply that endothelium-derived NO substantially contributes to the blood pressure decrease to PAF. However, it is still unclear whether the findings obtained with isolated blood vessels can be translated into the depressor response to PAF *in vivo* or whether EDHF contributes to the depressor response to PAF. The present study was performed to answer these questions in *in vivo* studies with anaesthetized rats.

## Methods

### *Measurement of systemic blood pressure and heart rate*

Male Wistar rats (SLC, Hamamatsu-City, Japan) weighing 200–400 g were anaesthetized with α-chloralose (80 mg $kg^{-1}$) and urethane (0.8 g $kg^{-1}$) given intraperitoneally. A left carotid artery was cannulated for the measurement of direct continuous systemic arterial blood pressure with a disposable pressure transducer (Model DTX*Plus* (DT-XX), Ohmeda Medical Devices Division Inc., Madison, WI, U.S.A.) through a carrier amplifier (Model AP-621G, Nihon Kohden, Tokyo, Japan). Mean arterial blood pressure was derived on-line from the phasic signals. Heart rate was monitored with a heart rate counter (Model AT-601G, Nihon Kohden, Tokyo, Japan) triggered by arterial pressure pulse. Both systemic arterial blood pressure and heart rate were recorded on a pen-writing recorder (WI-621G, Nihon Kohden, Tokyo, Japan). A cannula was also placed in the left and/or right femoral veins for administration of drugs (i.v.). The animals were allowed to spontaneously breathe through a tracheal cannula. Experiments were started when blood pressure and heart rate had stabilized.

### *Drugs*

The drugs used in the present study were as follows: PAF ($C_{16}$: 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine) (Bachem Feinchemikalien AG, Bubendorf, Switzerland); 2-[*N*-acetyl-*N*-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride (CV-6209), sodium nitroprusside (SNP) (Wako Pure Chemical, Osaka, Japan); $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Dojindo Laboratories, Kumamoto, Japan); L-arginine, urethane (Sigma, St. Louis, MO, U.S.A.); charybdotoxin (ChTX), apamin, endothelin-1 (ET-1) (Peptide Institute, Minoh-Shi, Osaka, Japan); acetylcholine chloride (ACh) (Daiichi Seiyaku, Tokyo, Japan); α-chloralose (Tokyo Kasei, Tokyo, Japan). PAF was stored in chloroform solution at −20°C. Just before usage of PAF, chloroform was evaporated with 100% $N_2$ gas and PAF solution was prepared with saline (0.9% NaCl) containing 2.5 mg $ml^{-1}$ bovine serum albumin (BSA) (Shigenobu *et al*., 1987).

### *Statistical analysis*

The data are presented as mean values ± s.e.mean and *n* refers to the number of experiments. Significance of differences between means was evaluated by paired or unpaired Student's *t*-test, unpaired Student's *t*-test with Welch's correction if necessary, or one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test. *P* values less than 0.05 were considered statistically significant.

## Results

### *Blood pressure decrease in response to PAF and its blockade by CV-6209*

The systemic mean arterial blood pressure and the heart rate in rats used in the present study were 117.1 ± 3.5 mmHg and 372 ± 7 beats/min ($n = 44$ for each), respectively.

PAF (1 μg $kg^{-1}$, i.v.) caused a decrease in arterial blood pressure within 1 min of infusion. The maximum arterial blood pressure change was attained within 5–10 min after the administration of PAF and it was about 60 mmHg (59.3 ± 4.6 mmHg, $n = 18$) in magnitude. The decrease in arterial blood pressure was gradually restored but full recovery was not attained even after 60 min (Figure 1). The blood pressure changes in response to PAF (1 μg $kg^{-1}$, i.v.) were completely blocked by a PAF receptor antagonist, CV-6209 (1 mg $kg^{-1}$, i.v.) (Figure 1). CV-6209 itself did not show any appreciable effects on the basal arterial blood pressure (before: 121.3 ± 18.0 mmHg *vs* after: 120.0 ± 13.2 mmHg, $n = 4$ for each, $P > 0.05$).

### *Effects of $N^G$-nitro-L-arginine methyl ester (L-NAME) on the depressor response to PAF*

To examine the contribution of NO to the depressor response to PAF, the effects of the NO synthase inhibitor L-NAME were examined. L-NAME (50 mg $kg^{-1}$, i.v.) itself elevated profoundly the systemic arterial blood pressure (Figure 2). The elevation of the arterial blood pressure due to L-NAME (50 mg $kg^{-1}$, i.v.) reached a maximum level within 5 min after its infusion and the change was by 82.5 ± 12.0 mmHg ($n = 4$) from 126.3 ± 23.8 mmHg to 208.8 ± 12.6 mmHg ($n = 4$ for each, $P < 0.01$). The elevation of systemic blood pressure by L-NAME lasted for at least 90 min. For instance, in a separate series of experiments, we observed that blood pressure level at 90 min after the administration of L-NAME (50 mg $kg^{-1}$, i.v.) was still higher than the blood pressure level before infusion of L-NAME (147.6 ± 10.9 mmHg *vs* 104.1 ± 4.3 mmHg, $n = 4$ for each, $P < 0.01$). The pressor response to L-NAME (50 mg $kg^{-1}$, i.v.) was significantly diminished by the pretreatment with L-arginine (1000 mg $kg^{-1}$, i.v.) for 10 min (Figure 2). L-Arginine (1000 mg $kg^{-1}$, i.v.) itself substantially lowered mean arterial blood pressure from 112.5 ± 6.6 mmHg to 76.3 ± 6.9 mmHg ($n = 4$ for each, $P < 0.05$).

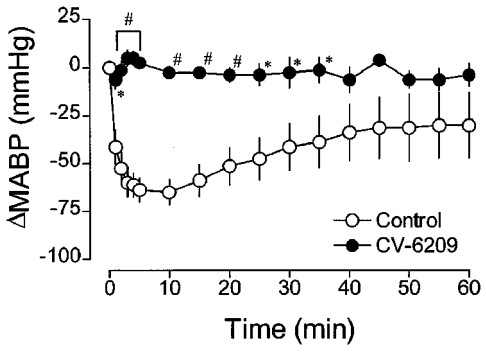


**Figure 1** Time courses of changes in mean arterial blood pressure (ΔMABP) after administration of PAF (1 μg $kg^{-1}$ i.v.) in control rats and animals treated with the PAF antagonist, CV-6209 (1 mg $kg^{-1}$, i.v.). CV-6209 was administered 20 min before infusion of PAF. Each point represents the mean ± s.e.mean for four observations. $^*P < 0.05$; $^\#P < 0.01$ show significant differences from control.

Figure 3 depicts typical experimental traces showing the effects of L-NAME on the arterial blood pressure decrease in response to PAF. L-NAME (50 mg $kg^{-1}$, i.v.) was administered into rats 20 min before infusion of PAF (1 $\mu$g $kg^{-1}$, i.v.). As shown in Figure 3, pretreatment of rats with L-NAME did not diminish the depressor response to PAF. On the contrary, the blood pressure decrease in response to PAF was enhanced in the rat pretreated with L-NAME (Figure 3B). Figure 4A shows the summarized time courses of mean arterial blood pressure changes after administration of PAF (1 $\mu$g $kg^{-1}$, i.v.) in rats treated with L-NAME (50 mg $kg^{-1}$, i.v.). Enhancement of the blood pressure decrease in response to PAF was also observed when the basal blood pressure was elevated by endothelin-1 (5 $\mu$g $kg^{-1}$, i.v., administered 15 min before PAF) by about 80 mmHg (78.3 $\pm$ 1.7 mmHg, $n=3$). Figure 4B shows the relationships between the initial arterial blood pressure levels and the changes in arterial blood pressure due to PAF, and these parameters were in a good correlation ($r^2=0.7433$).

Since the extent of the blood pressure decrease in response to PAF was found to be affected strongly by the basal blood pressure level (Figure 4), depressor responses to PAF in the absence and presence of L-NAME were normalized against the blood pressure decreases to the NO donor sodium nitroprusside (SNP; 10 $\mu$g $kg^{-1}$, i.v.). As shown in Figure 5, when the depressor responses to PAF, in the absence and presence of L-NAME, were normalized in this way, no significant difference was observed ($n=4$; Figure 5B). The

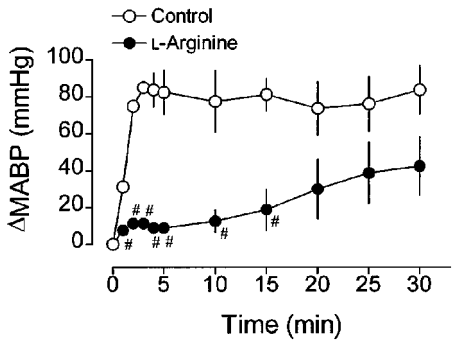


**Figure 2** Time courses of changes in mean arterial blood pressure (ΔMABP) after administration of L-NAME (50 mg $kg^{-1}$, i.v.) in control rats and animals treated with L-arginine (1000 mg $kg^{-1}$, i.v.). L-Arginine was administered 10 min before infusion of L-NAME. Each point represents the mean $\pm$ s.e.mean for four observations. $^{\#}P<0.01$ shows significant differences from control.

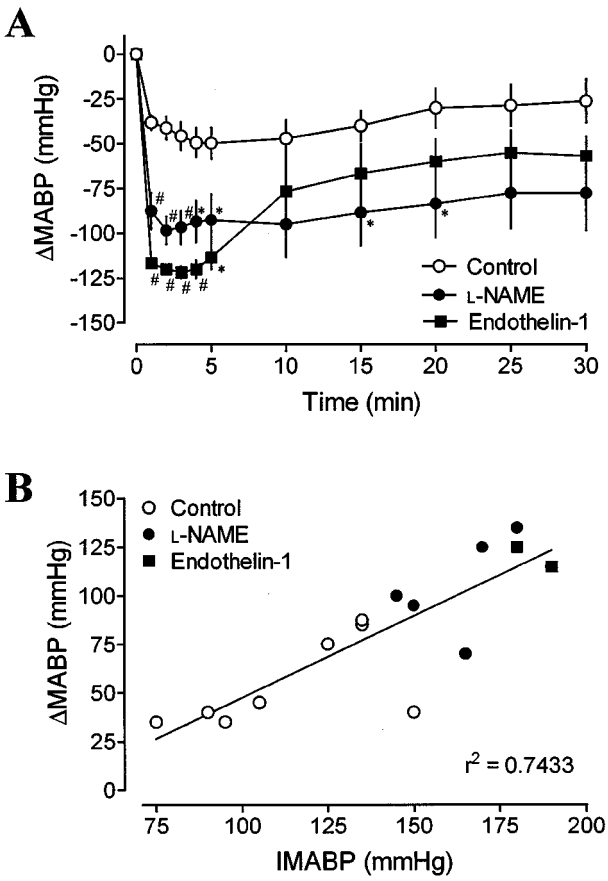


**Figure 4** Effects of L-NAME and endothelin-1 on systemic arterial blood pressure depression produced by PAF in anaesthetized rats. (A) Time courses of changes in mean arterial blood pressure (ΔMABP) after administration of PAF (1 $\mu$g $kg^{-1}$, i.v.) in control rats and animals treated with L-NAME (50 mg $kg^{-1}$, i.v.) or endothelin-1 (5 $\mu$g $kg^{-1}$, i.v.). L-NAME or endothelin-1 was administered 15–20 min before infusion of PAF. Each point represents the mean $\pm$ s.e.mean for three to eight observations. $^{*}P<0.05$; $^{\#}P<0.01$ show significant differences from control. (B) Relationship between initial mean arterial blood pressure (IMABP) and ΔMABP elicited by infusion of PAF (1 $\mu$g $kg^{-1}$, i.v.). Each point represents the peak depressor response.

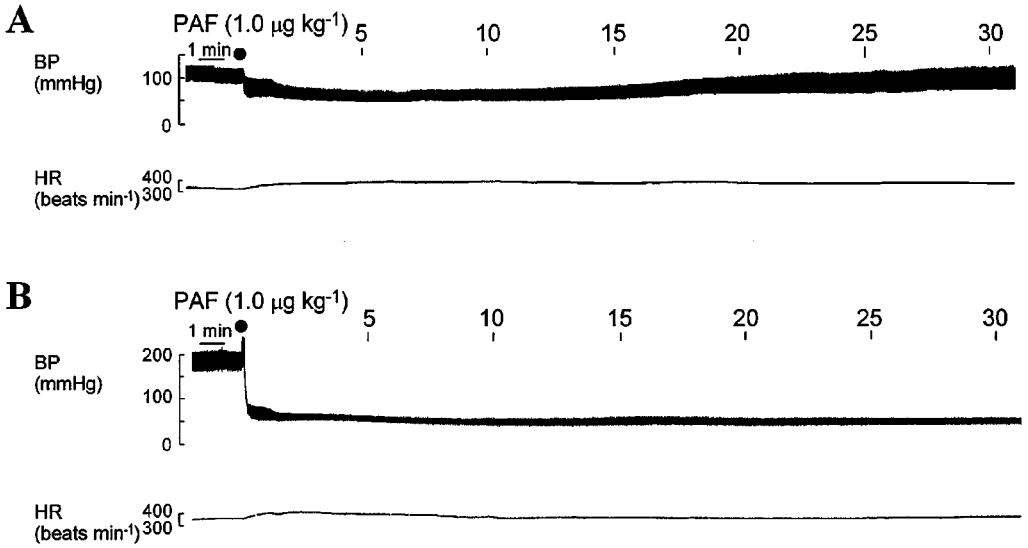


**Figure 3** Typical traces showing the lack of effect of L-NAME (50 mg $kg^{-1}$, i.v.) on the systemic arterial blood pressure (BP) changes produced by PAF (1 $\mu$g $kg^{-1}$, i.v.) in anaesthetized rats. L-NAME was administered 20 min before infusion of PAF (B).

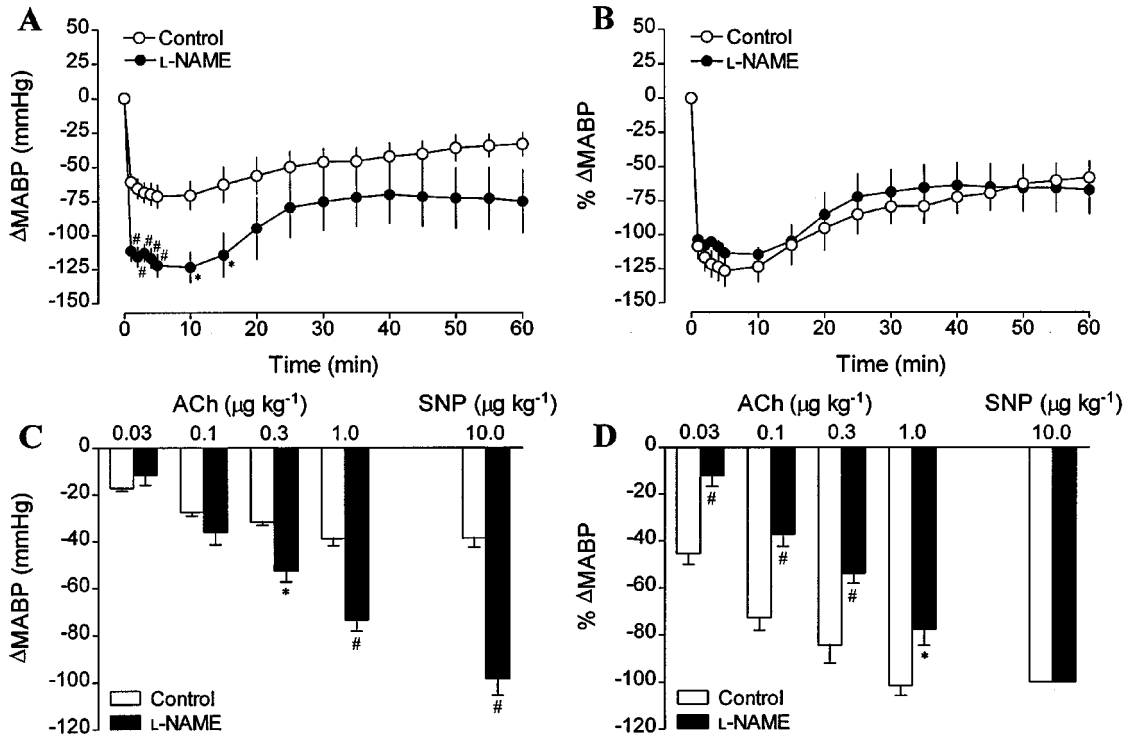


**Figure 5** Effect of L-NAME (50 mg $kg^{-1}$, i.v.) on depressor responses to PAF (1 $\mu$g $kg^{-1}$, i.v.) and ACh (0.03–1 $\mu$g $kg^{-1}$, i.v.). Data are normalized against the blood pressure decrease evoked by SNP (10 $\mu$g $kg^{-1}$, i.v.). L-NAME was administered 20 min before infusion of PAF, SNP or ACh. (A) Time courses of changes in mean arterial blood pressure (ΔMABP) after administration of PAF in control rats and animals treated with L-NAME. Each point represents the mean ± s.e.mean for four observations. $^{*}P<0.05$; $^{\#}P<0.01$ show significant differences from control. (B) Time course of normalized blood pressure decrease in response to infusion of PAF in control rats and animals treated with L-NAME. Each point represents the mean ± s.e.mean for four observations. (C) ΔMABP after administration of ACh in control rats and animals treated with L-NAME. Each column represents the mean ± s.e.mean for five observations. $^{*}P<0.05$; $^{\#}P<0.01$ show significant differences from control. (D) Normalized depressor responses to ACh (0.03–1 $\mu$g $kg^{-1}$, i.v.) in control rats and animals treated with L-NAME. Each column represents the mean ± s.e.mean for five observations. $^{*}P<0.05$; $^{\#}P<0.01$ show significant differences from control.

blood pressure decrease in response to ACh (0.03–1.0 $\mu$g $kg^{-1}$, i.v.) was also enhanced in rats treated with L-NAME (Figure 5C) although a significant attenuation was revealed when these responses were normalized against depressor responses to SNP ($n=5$; Figure 5D)

### *Effects of combination treatment with charybdotoxin plus apamin on the depressor response to PAF*

In a separate series of experiment, effects of combination treatment with charybdotoxin plus apamin on the depressor response to PAF were examined to investigate the possible role of EDHF in the blood pressure changes caused by PAF. Charybdotoxin (0.4 mg $kg^{-1}$, i.v.) and apamin (0.2 mg $kg^{-1}$, i.v.) were administered simultaneously into rats 20 min before infusion of PAF (1 $\mu$g $kg^{-1}$, i.v.). Basal blood pressure level was elevated immediately after the infusion of charybdotoxin plus apamin, and it reached a maximum level within 2 min after the toxin infusion. The change of the basal blood pressure due to the treatment with both toxins was by 44.5 ± 4.0 mmHg ($n=5$) (from 128.0 ± 6.8 mmHg to 172.5 ± 8.7 mmHg, $n=5$, $P<0.01$). However, in contrast to the pressor effect of L-NAME, pressor response to charybdotoxin plus apamin was not sustained, and declined to the similar blood pressure level as that of control (130.0 ± 11.1 mmHg, $n=5$ for each, $P>0.05$) at 20 min after the toxin administration.

Figure 6 depicts the typical traces showing the effects of charybdotoxin plus apamin on the blood pressure decrease in response to PAF. As shown in Figure 6, the recovery of depressed blood pressure due to PAF was more progressive in rats treated with both peptide toxins than that in untreated rats. Furthermore, the immediate depressor response observed within 1 min after PAF infusion was also substantially suppressed in the rat treated with charybdotoxin plus apamin (Figure 6B). Summarized time courses of the blood pressure changes after administration of PAF (1 $\mu$g $kg^{-1}$, i.v.) in the absence and presence of charybdotoxin plus apamin were shown in Figure 7A,B. In contrast, the depressor response to SNP (1 mg $kg^{-1}$, i.v.) was not significantly affected by the treatment with charybdotoxin plus apamin (Figure 7C,D). Charybdotoxin (0.4 mg $kg^{-1}$, i.v.) or apamin (0.2 mg $kg^{-1}$, i.v.) alone did not substantially affect the blood pressure decrease in response to PAF (1 $\mu$g $kg^{-1}$, i.v.) (Figure 8).

The effects of charybdotoxin itself on the basal blood pressure were complicated. After infusion of charybdotoxin (0.4 mg $kg^{-1}$, i.v.), basal blood pressure was increased by 17.5 ± 6.6 mmHg ($n=4$) from 110.4 ± 5.9 mmHg to 127.9 ± 10.3 mmHg ($n=4$, $P>0.05$) at 1 min, and then transiently decreased to 94.9 ± 12.1 mmHg ($n=4$, $P>0.05$) at 2 min. Thereafter, basal blood pressure was restored to 131.3 ± 14.7 mmHg ($n=4$, $P>0.05$) at 10 min after its administration, and the value was 121.9 ± 12.6 mmHg ($n=4$ for each, $P>0.05$) just before infusion of PAF (1 $\mu$g $kg^{-1}$, i.v.). On the other hand, apamin (0.2 mg $kg^{-1}$, i.v.) itself transiently elevated basal blood pressure by about 10 mmHg (8.8 ± 3.6 mmHg, $n=4$) at 1 min after its administration, but basal blood pressure level just before infusion of PAF (1 $\mu$g $kg^{-1}$, i.v.) was not significantly different from the value before administration of apamin (116.3 ± 3.2 mmHg *vs* 118.1 ± 1.9 mmHg, $n=4$ for each, $P>0.05$).

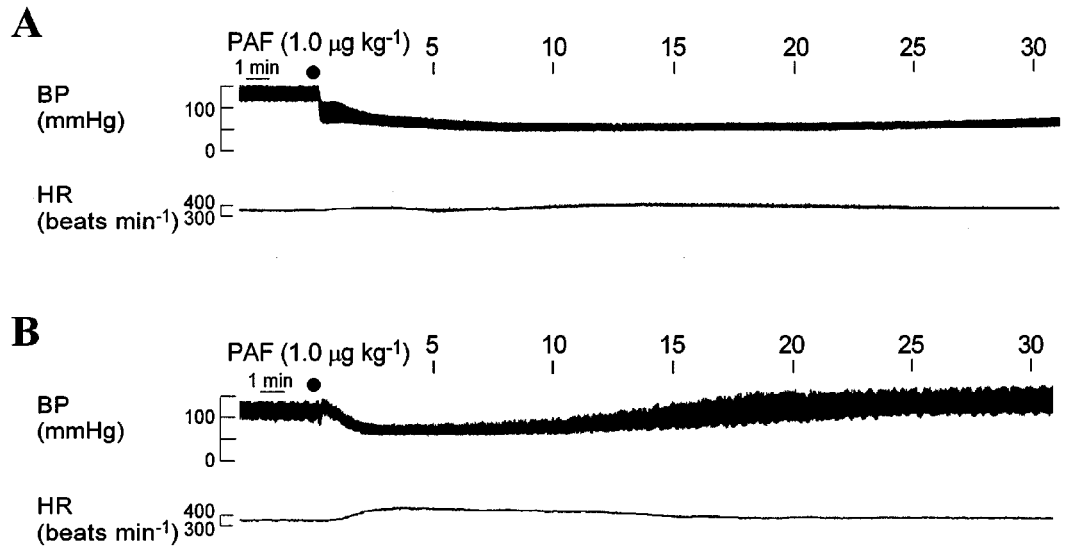


**Figure 6** Typical traces showing the effect of charybdotoxin (0.4 mg $kg^{-1}$, i.v.) and apamin (0.2 mg $kg^{-1}$, i.v.) on the systemic arterial blood pressure (BP) changes produced by PAF (1 $\mu$g $kg^{-1}$, i.v.) in anaesthetized rats. Charybdotoxin and apamin were simultaneously administered 20 min before infusion of PAF (B).

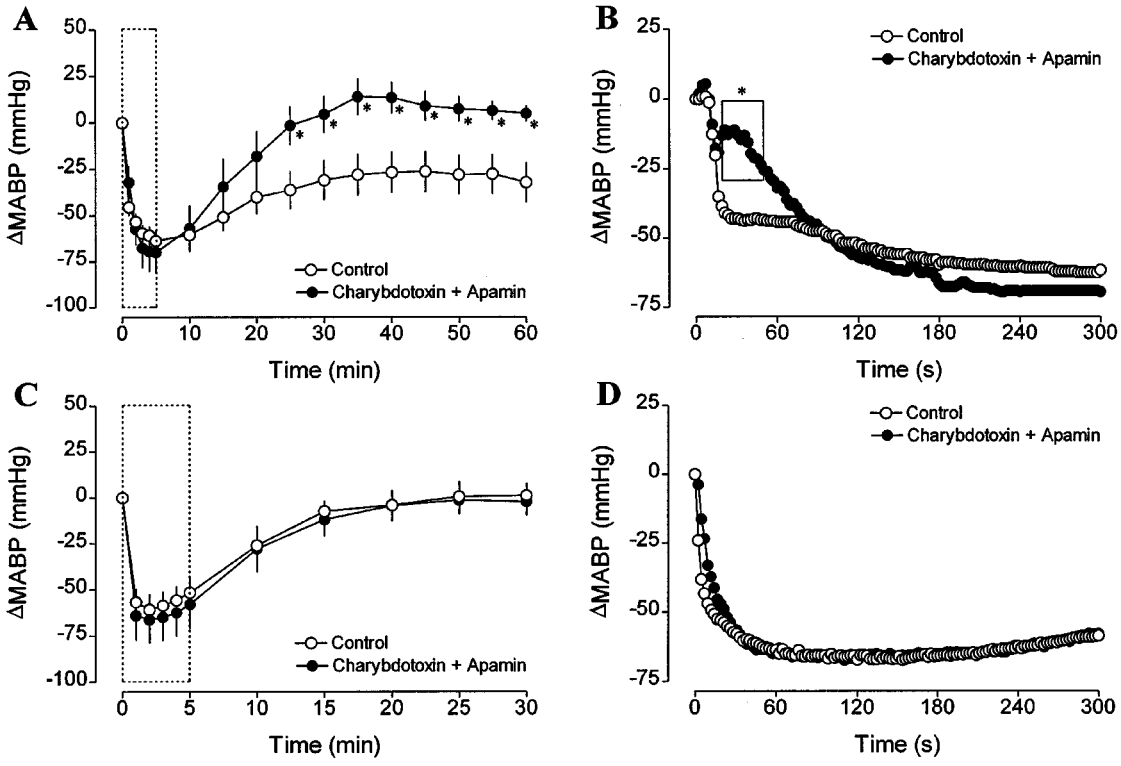


**Figure 7** Effect of charybdotoxin (0.4 mg $kg^{-1}$, i.v.) and apamin (0.2 mg $kg^{-1}$, i.v.) on depressor responses to PAF (1 $\mu$g $kg^{-1}$, i.v.) and SNP (1 mg $kg^{-1}$, i.v.) in anaesthetized rats. Charybdotoxin and apamin were simultaneously administered 20 min before infusion of PAF or SNP. (A) Time courses of changes in mean arterial blood pressure ($\Delta$MABP) after administration of PAF in control rats and animals treated with charybdotoxin and apamin. Each point represents the mean$\pm$s.e.mean for five to six observations. *$P<0.05$ shows significant differences from control. (B) Expanded time course to show $\Delta$MABP within 5 min of administration of PAF in control rats and animals treated with charybdotoxin and apamin. All data are from (A) and only mean values are shown. *$P<0.05$ shows significant differences from control. (C) Time courses of $\Delta$MABP after administration of SNP in control rats and animals treated with charybdotoxin and apamin. Each point represents the mean$\pm$s.e.mean for three to four observations. (D) Expanded time course to show $\Delta$MABP within 5 min of administration of SNP in control rats and animals treated with charybdotoxin and apamin. All data are from (C) and only mean values are shown.

## Discussion

The aim of the present study was to elucidate the roles of NO and EDHF in the blood pressure decrease in response to PAF in anaesthetized rats. The findings suggest that EDHF contributes to the depressor response to PAF, and NO plays a less pronounced role in this response as compared to its significant role in the blood pressure decrease to ACh.

L-Arginine derivatives including L-NAME are widely used in *in vitro* studies as specific inhibitors of NO synthase to examine the role of NO in relaxant responses of isolated blood vessels to a wide variety of vasoactive substances.

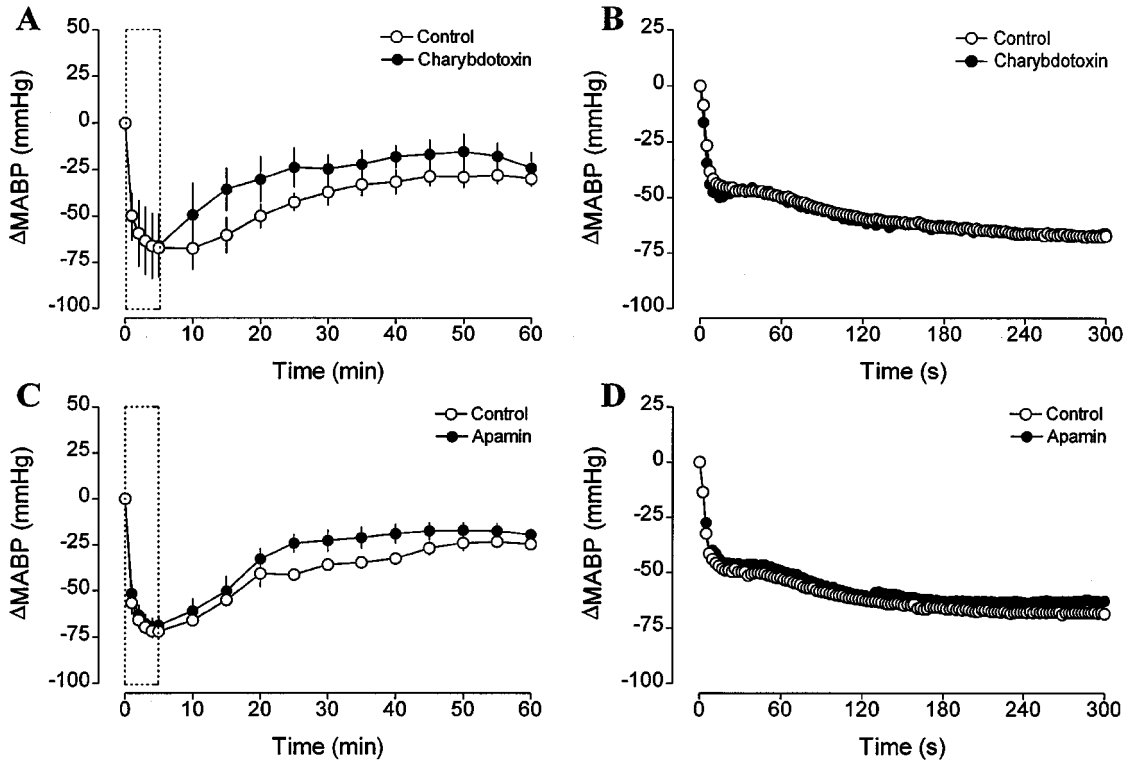


**Figure 8** Effect of charybdotoxin (0.4 mg $kg^{-1}$, i.v.) and apamin (0.2 mg $kg^{-1}$, i.v.) on depressor responses to PAF (1 $\mu$g $kg^{-1}$, i.v.) in anaesthetized rats. Charybdotoxin and apamin were administered 20 min before infusion of PAF. (A) Time courses of changes in mean arterial blood pressure (ΔMABP) after administration of PAF in control rats and animals treated with charybdotoxin. Each point represents the mean ± s.e.mean for three to four observations. (B) Expanded time course to show ΔMABP within 5 min of administration of PAF in control rats and animals treated with charybdotoxin. All data are from (A) and only mean values are shown. (C) Time courses of ΔMABP after administration of PAF in control rats and animals treated with apamin. Each point represents the mean ± s.e.mean for four observations. (D) Expanded time course to show ΔMABP within 5 min of administration of PAF in control rats and animals treated with apamin. All data are from (C) and only mean values are shown.

Furthermore, they have been also used in *in vivo* studies to determine the roles of NO in the regulation of systemic blood pressure (Aisaka *et al*., 1989a,b; Gardiner *et al*., 1990; Nakahara *et al*., 1999; Rees *et al*., 1989; 1990; van Gelderen *et al*., 1991). In the present study, L-NAME (50 mg $kg^{-1}$) elicited a long-lasting potent pressor response in anaesthetized rats (Figure 2). As the pressor action of L-NAME was significantly diminished by the NO precursor, L-arginine (1000 mg $kg^{-1}$), the systemic arterial blood pressure elevation by L-NAME is a result of the inhibition of NO synthesis. These findings indicate that NO plays an important role as the determinant factor to regulate the basal systemic blood pressure level, and supports the results in previous reports (Aisaka *et al*., 1989b; Rees *et al*., 1989; 1990; van Gelderen *et al*., 1991).

The evidence obtained in *in vitro* studies with isolated blood vessels imply that dilatation of peripheral blood vessels due to the activation of NO-cyclic GMP system partly accounts for the blood pressure decrease due to PAF (Chiba *et al*., 1990; Kamata *et al*., 1996a; Moritoki *et al*., 1992). Since the depressor response to PAF was eliminated by a PAF antagonist, CV-6209, an activation of PAF-specific receptor(s) mediates the depressor response to this phospholipid. On the other hand, our present *in vivo* study showed that the blood pressure decrease in response to PAF was not diminished by L-NAME, and thus do not support the idea that NO is an essential factor in the depressor response to PAF. L-NAME at a similar dose (30 mg $kg^{-1}$) as that used in the present study (50 mg $kg^{-1}$) caused almost the maximum change in blood pressure elevation in anaesthetized rats (Rees *et al*., 1990), and thus most of the NO production should be abolished in our experimental condition. Furthermore, PAF-produced depressor response normalized against the depressor response to SNP was not significantly different in rats treated without and with L-NAME (50 mg $kg^{-1}$) whereas ACh-produced blood pressure decrease was substantially diminished in rat treated with L-NAME (50 mg $kg^{-1}$) as compared to the depressor response to SNP. These observations obtained in *in vivo* studies suggest that NO-cyclic GMP system plays less of a determinant role in the depressor response to PAF as compared to the regulation of basal blood pressure level.

NO has been suggested to play a significant role in reductions in blood pressure to vasoactive substances other than PAF such as ACh and bradykinin (Rees *et al*., 1990). However, other studies have suggested roles for other factors (Aisaka *et al*., 1989a; Gardiner *et al*., 1990; Nakahara *et al*., 1999; van Gelderen *et al*., 1991). In relation to this matter, Nakahara *et al*. (1999) recently reported that reduced endothelial NO production is not a major mechanism responsible for the suppression of ACh-produced depressor response to NO synthase inhibitor, $N^G$-nitro-L-arginine (L-NNA) because of the following reasons: (1) Combination treatment with L-NNA plus hydralazine diminished L-NNA-induced suppression of ACh-produced blood pressure decrease; (2) Inhibition by L-NNA was less pronounced for carbachol-induced depressor response than for ACh-induced response. Thus, the elevation of blood pressure caused by L-NNA itself, but not reduced NO production, may have more important role in attenuating the depressor response to ACh by this NO synthase inhibitor (Nakahara *et al*., 1999).

EDHF(s) (Garland *et al*., 1995; Mombouli & Vanhoutte, 1997; Taylor & Weston, 1988) is a proposed EDRF which may mediate non-NO- and non-prostacyclin ($PGI_2$)-mediated endothelium-dependent vascular relaxations. The chemical identity of this putative diffusible factor has not yet been established though some EDHF candidates have been proposed so far (Bauersachs *et al*., 1994; Edwards *et al*., 1998; Randall & Kendall, 1998). Another explanation for non-NO-, non-$PGI_2$-mediated endothelium-dependent vascular relaxations is that they are mediated *via* non-diffusible factor(s) (Yamamoto *et al*., 1998). Nevertheless, it is widely recognized that non-NO-, non-$PGI_2$-mediated relaxant responses are totally abolished by the combination treatment with charybdotoxin plus apamin in *in vitro* studies with isolated blood vessels (Edwards *et al*., 1998; Yamanaka *et al*., 1998). Our present *in vivo* findings showed that charybdotoxin plus apamin treatment substantially diminished PAF-produced depressor response. The doses of charybdotoxin (0.4 mg $kg^{-1}$) and apamin (0.2 mg $kg^{-1}$) used in the present study are roughly estimated to correspond to $10^{-7}-10^{-6}$ M, which are almost equal to the concentrations employed in *in vitro* studies with isolated tissues. Furthermore, (1) combination treatment with charybdotoxin plus apamin did not affect the depressor response to SNP, the activation of which is thought to be unrelated to EDHF-mediated mechanism, and (2) charybdotoxin or apamin itself did not affect the depressor response to PAF. Therefore, the combination treatment with charybdotoxin plus apamin seems to be selective for the blockade of EDHF-mediated component in the blood pressure decrease in response to PAF. Thus, we would like to propose that EDHF substantially contributes to the depressor action of PAF. Our present study is the first attempt to elucidate the role of EDHF in the systemic blood pressure regulation in *in vivo* studies.

Diameter changes in small resistance vessels such as arterioles seem to partly contribute to systemic arterial blood pressure decrease in response to PAF. Pharmaco-mechanical and biochemical evidence shows that the role of EDHF(s) is more important than NO in the resistance blood vessels with smaller diameter whereas the contribution of NO is greater in conduit blood vessels with larger diameter (Garland *et al*., 1995; Nagao *et al*., 1992; Shimokawa *et al*., 1996). Therefore, mechanical changes of large blood vessels in response to PAF observed in *in vitro* studies (Chiba *et al*., 1990; Kamata *et al*., 1996a) may be influenced merely by NO rather than EDHF, which would be an explanation for the discrepancy between the results obtained from the present *in vivo* study and those with isolated vascular preparations.

Significant inhibition of the depressor response to PAF by charybdotoxin plus apamin was attained at the initial phase within 1 min after the administration of PAF and also at the sustained phase of the depressor response to PAF (Figure 6 and Figure 7A,B). At present, we do not have any clear explanations for this biphasic inhibition of PAF-produced depressor response by these putative EDHF inhibitors. One feasible explanation would be that EDHF(s) is not a single factor (substance) but consists of multiple diffusible factors. Contribution of each EDHF to the depressor effect of PAF may differ during the time course of the development of depressor response to PAF. Actually, a wide variety of substances or mechanisms have been proposed as EDHF candidates (Edwards *et al*., 1998; Fisslthaler *et al*., 1999; Randall & Kendall, 1998; Taylor *et al*., 1998; Yamamoto *et al*., 1998). Another possibility would be the existence of PAF receptor subtypes, activations of which contribute differentially to the development of PAF-produced depressor response.

As compared to the action of charybdotoxin itself or apamin alone, combination treatment of charybdotoxin plus apamin significantly and potently elevated basal blood pressure though the pressor action due to the toxin treatment was not sustained. These findings may imply that EDHF as well as NO partly contributes to the regulation of basal blood pressure level *in vivo*. However, at present, we cannot exactly know which is the more determinant factor for the control of basal blood pressure level, NO or EDHF. We also cannot give any reasonable explanations to the transient characteristic of basal blood pressure elevation by charybdotoxin plus apamin. It could be possible that charybdotoxin and apamin are inactivated by peptidases after administration into rats. To answer the above question and confirm the role of EDHF in the basal blood pressure regulation, other non-peptide like EDHF inhibitors would be necessary.

All of the previous studies on NO- and $PGI_2$-independent (EDHF-mediated) endothelium-dependent functional changes have been performed with isolated vascular preparations. Our present *in vivo* study is the first attempt to elucidate the physiological significances of EDHF and suggests the role of this putative endothelium-derived factor in the depressor effect of PAF. On the other hand, in the depressor response to PAF, multiple factors other than the dilatation of resistance arteries seem to be involved (Bessin *et al*., 1983; Laurindo *et al*., 1989; King *et al*., 1995; Yamanaka *et al*., 1992). Since the depressor response to PAF and the endothelium-dependent relaxation by PAF are not influenced by indomethacin in rats (Kamata *et al*., 1989; Tanaka *et al*., 1983), contribution of vasodilator prostaglandins seems to be minor in the action of PAF at least in rats. However, the possible role of other metabolites of arachidonic acid should be also considered in the depressor response to PAF and should await further elucidation in future.

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