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Involvement of platelet cyclic GMP but not cyclic AMP suppression in leukocyte-dependent platelet adhesion to endothelial cells induced by platelet-activating factor *in vitro*

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1 Incubation of endothelial cells with platelets in the absence or the presence of PAF (10 nM) markedly increased platelet cyclic AMP levels, which were significantly decreased by indomethacin (3 μM). Co-incubation of endothelial cells and platelets with polymorphonuclear leukocytes (PMNs) did not change the platelet cyclic AMP levels.

2 Incubation of endothelial cells with platelets in the absence of PAF increased platelet cyclic GMP levels, which were increased 3.5 fold by PAF. These cyclic GMP levels were significantly decreased by N^G-nitro-L-arginine (100 μM), and completely by methylene blue (10 μM). When endothelial cells and platelets were co-incubated with PMNs, the cyclic GMP level in the cell mixture was 42.5 and 65.3% lower than that in endothelial cells and platelets without and with PAF stimulation, respectively.

3 PAF induced platelet adhesion to endothelial cells only when PMNs were present. Methylene blue dose-dependently potentiated the PMN-dependent platelet adhesion induced by PAF, although it had no effect in the absence of PMNs.

4 Sodium nitroprusside and 8-bromo cyclic GMP but not dibutyryl cyclic AMP significantly, although partially, inhibited the platelet adhesion. Inhibition of cyclic GMP-specific phosphodiesterase by zaprinast slightly inhibited the PMN-induced platelet adhesion and potentiated the inhibitory effect of 8-bromo cyclic GMP, while these drugs markedly inhibited the adhesion of platelet aggregates induced by PMN sonicates.

5 These results suggest that the impairment by activated PMNs of EDRF-induced platelet cyclic GMP formation is involved in part in the mechanism of PMN-dependent platelet adhesion to endothelial cells induced by PAF *in vitro*. The precise mechanism still remains to be clarified.

Keywords: Platelet adhesion; endothelial cells; leukocytes; EDRF; cyclic AMP; cyclic GMP; platelet-activating factor

Introduction

Platelets do not adhere to normal endothelial surfaces. This highly anti-thrombogenic property of the endothelium can be attributed to its ability to synthesize and release a variety of substances, including prostacyclin and endothelium-derived relaxing factor (EDRF) (Moncada & Vane, 1978; Azuma *et al.*, 1986; Furlong *et al.*, 1987). These mediators inhibit platelet aggregation and/or adhesion via adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation, respectively (Moncada & Vane, 1978; Radomski *et al.*, 1987; Sneddon & Vane, 1988; Venturini *et al.*, 1992). However, in some *in vivo* experimental models in which animals were injected with ADP (Fujimoto *et al.*, 1985), arachidonate (Fujimoto *et al.*, 1988) or platelet-activating factor (PAF; Vargaftig & Bourgain, 1989), platelets can adhere to 'injured' endothelium as well as to the site of exposure of sub-endothelium. In some *in vitro* experiments, platelets can also adhere to endothelial cells that have been virally infected (Curwen *et al.*, 1982) or stimulated with thrombin (Czervionke *et al.*, 1978; Kaplan *et al.*, 1989). These platelet adhesions are presumed to accompany the loss of anti-thrombogenic properties of the endothelium. However, it is unclear how prostacyclin and EDRF are involved in these mechanisms.

In a previous paper, we demonstrated that PAF induced platelet adhesion to endothelial cells in the presence of PMNs *in vitro* and had no effect in the absence of PMNs (Hirafuji & Shinoda, 1991a). N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) also induced the platelet adhesion via the mechanism dependent on PMN-derived PAF (Hirafuji & Shinoda, 1991b). Since superoxide dismutase significantly prevented the platelet adhesion, superoxide anions derived from activated PMNs seem to have a role in the ultimate process inducing the platelet-endothelial cell interaction (Hirafuji & Shinoda, 1993). In the present study, to evaluate the involvement of cyclic AMP and cyclic GMP in the mechanism of platelet adhesion, we measured the cyclic AMP and cyclic GMP levels during the intercellular interaction, and determined the effects of drugs which alter cellular levels of cyclic nucleotides on the platelet adhesion.

Methods

Endothelial cells, platelets and PMNs

Endothelial cells, platelets and PMNs were prepared as described previously (Hirafuji & Shinoda, 1991a). Briefly, primary endothelial cells isolated from human umbilical cord veins were grown in 24-well plates (Primaria, Falcon) until confluent (4-6 days) in Ham's F-12 medium containing 15% foetal calf serum, 25 μg ml^{-1} endothelial cell mitogen, 90 μg ml^{-1} heparin, 50 u ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Just prior to experiments, endothelial cells were washed with Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4) and 0.25% bovine serum albumin (BSA; HBSS). Washed rabbit platelets were finally resuspended in HBSS at 5×10^8 platelets ml^{-1}. Radiolabelled platelets were

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obtained by incubation with 0.5 μCi ml^{-1} [^{3}H]-adenine for 30 min (Curwen *et al.*, 1982). After washing out free radioactivity, cold adenine (100 μM) was added to the final platelet suspension. Rabbit PMNs were finally suspended in calcium- and magnesium-free HBSS at 1×10^7 cells ml^{-1}. Calcium (1.3 mM) and magnesium (1.0 mM) were added just prior to the experiments. In some experiments, PMN suspension was stimulated with PAF (26.7 nM) for 10 min at 37°C, and sonicated for 20 s at 0°C to obtain PMN sonicates.

Determination of cyclic AMP and cyclic GMP

Following incubation of endothelial cells, platelets (7.5×10^7 cells per well) and/or PMNs (7.5×10^5 cells per well) for 20 min at 37°C, the reaction was stopped by addition of cold trichloroacetic acid (TCA; 6%). The medium was transferred to vials, and the cells were extracted again with 6% TCA. The extracts were centrifuged, and TCA was removed from the supernatants by washing it four times with five volumes of water-saturated diethylether. Samples were then frozen and lyophilized. After reconstitution with a buffered solution, cyclic AMP and cyclic GMP were determined with enzyme-immunoassay kits (BioTrak, Amersham).

Platelet adhesion

Radiolabelled platelets (7.5×10^7 cells per well) and/or PMNs (7.5×10^5 cells per well) were added to endothelial cell monolayers, and incubated for 20 min at 37°C with gentle shaking. In some experiments, platelets and PMN sonicates were incubated with endothelial cells that had been pretreated with 500 μM aspirin and 300 μM N^G-nitro-L-arginine (L-NOARG) for 15 min. Following incubation, the wells were gently washed with HBSS to remove non-adherent cells. Triton X-100 (1%) was added to each well to lyse the remaining cells, and the radioactivity in the wells was counted. Platelet adhesion was expressed as a percentage of the total radioactivity in cell suspension added to the wells (Hirafuji & Shinoda, 1991a).

Materials

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), N^G-nitro-L-arginine, adenine, dibutyryl cyclic AMP and 8-bromo cyclic GMP were purchased from Sigma; Ham's F-12 culture medium, foetal calf serum, penicillin and streptomycin from Gibco; endothelial mitogen from Biomedical Technologies Inc.; Lymphoprep (9.6% sodium metrizoate and 5.6% Ficoll) from Nycomed AS; sodium nitroprusside dihydrate, methylene blue and HEPES from Wako Chemicals; aspirin (DL-lysin salt) from Midorijuhji Co.; Hanks' balanced salt solution from Nissui Pharmaceut.; [^{3}H]-adenine (24 Ci mmol^{-1}) from Amersham, Buckinghamshire. Zaprinast was a gift from Rhône-Poulenc Rorer, Ltd.

Statistical analysis

Results are expressed as mean±s.e.mean of n experiments performed in triplicate or of replicate determinations. A representative result was demonstrated as to the levels of cyclic nucleotides since the basal levels and responses to PAF were very variable depending on the experiment, possibly due to the variation in batches of human endothelial cells. The experiments were repeated at least three times with essentially similar results. Statistical analysis was made by Student's t test, and P values less than 0.05 were considered as significant.

Results

Cyclic AMP levels

Figure 1 shows a representative result with cyclic AMP levels after incubation of endothelial cells and platelets in the absence or the presence of PAF (10 nM). Basal cyclic AMP levels in endothelial cells and platelets were 0.12±0.003 pmol per well and 0.38±0.01 per 7.5×10^7 cells, mean±s.e.mean of triplicate, respectively, and were not significantly increased by PAF stimulation. When endothelial cells were co-incubated with platelets in the absence of PAF, the cyclic AMP level in the incubation mixture was markedly higher (2.68±0.17 pmol per well) than that observed with either type of cell alone. The cyclic AMP level was slightly increased by PAF to 3.01±0.15 pmol per well. Indomethacin (3 μM) significantly ($P<0.01$) inhibited the increase in cyclic AMP levels induced by co-incubation of endothelial cells and platelets in the absence and the presence of PAF by 85.7 and 75.1%, respectively.

The basal cyclic AMP level in PMNs was 0.15±0.01 pmol per 7.5×10^5 cells, and was unchanged by PAF stimulation. In contrast to the incubation with platelets, incubation of endothelial cells with PMNs did not cause a synergistic increase in the cyclic AMP level without or with PAF (not shown). As shown in Figure 2, when endothelial cells and platelets were co-incubated with PMNs, the basal cyclic AMP level in the cell

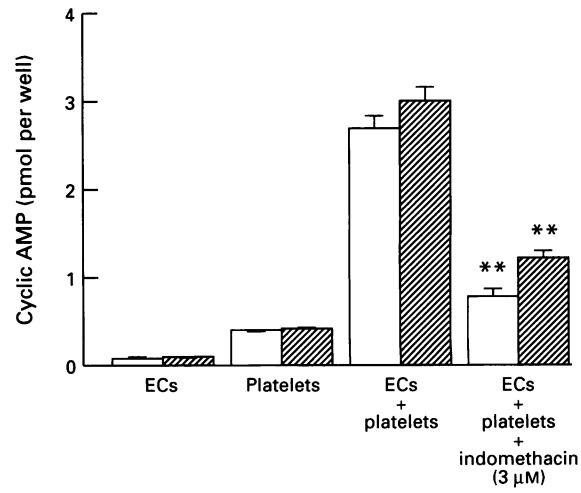

Figure 1 Effect of indomethacin on cyclic AMP formation by the interaction of endothelial cells and platelets. Endothelial cells without or with platelets were incubated in the absence (open columns) or the presence of 10 nM PAF (hatched columns) for 20 min at 37°C. At the end of incubation, cyclic AMP was extracted and determined by enzyme-immunoassay. Mean±s.e.mean of a representative of 3 experiments performed in triplicate. **$P<0.01$ vs ECs + platelets.

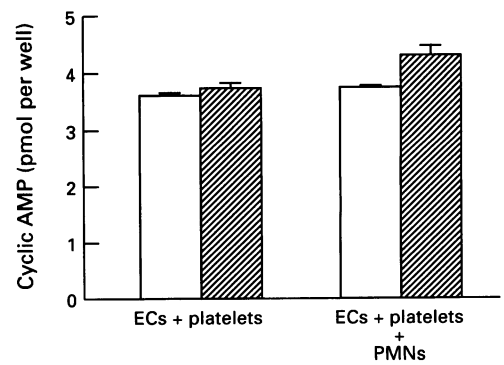

Figure 2 Cyclic AMP formation by interactions of endothelial cells, platelets and PMNs. Endothelial cells and platelets without or with PMNs were incubated in the absence (open columns) or the presence of PAF (hatched columns) for 20 min at 37°C. Mean±s.e.mean of a representative of 3 experiments performed in triplicate.

mixture (3.73±0.03 pmol per well) was not significantly higher than that with endothelial cells and platelets (3.59±0.06 pmol per well). The cyclic AMP level in these cell mixtures was slightly increased by PAF stimulation (4.26±0.2 pmol per well).

Cyclic GMP levels

Figure 3 demonstrates a representative result showing cyclic GMP levels after the incubation of endothelial cells and platelets in the absence or the presence of PAF. Basal cyclic GMP levels in endothelial cells and platelets were 0.31±0.01 pmol per well and 0.68±0.12 pmol per 7.5×10^7 cells, respectively, and were not significantly increased by PAF stimulation. When endothelial cells were co-incubated with platelets in the absence of PAF, the cyclic GMP level was markedly higher (4.62±0.23 pmol per well) than that in either type of cell alone. PAF stimulation caused a further 3.5 fold increase in the cyclic GMP level to 15.97±0.83 pmol per well. L-NOARG (100 μM) significantly ($P<0.01$) inhibited the increase in cyclic GMP levels induced by co-incubations of endothelial cells and platelets without and with PAF by 59.5 and 38.1%, respectively. Methylene blue (10 μM) also completely suppressed cyclic GMP levels without and with PAF by 91.7 and 98.5%, respectively.

Basal cyclic GMP level in PMNs (0.02±0.001 pmol per 7.5×10^5 cells) was near the detection limit of our assay, and was unchanged by PAF stimulation. The incubation of endothelial cells with PMNs did not cause a synergistic increase in cyclic GMP level without or with PAF (not shown). As shown in Figure 4, in contrast to cyclic AMP, when endothelial cells and platelets were co-incubated with PMNs, the basal cyclic GMP level in these cell mixtures (2.11±0.20 pmol per well) was 42.5% lower than that in endothelial cells and platelets (3.67±0.16 pmol per well). PAF induced a 2.1 fold increase in the cyclic GMP level in these cell mixtures (4.34±0.68 pmol per well). However, this cyclic GMP level was 65.3% lower than that in endothelial cells and platelets with PAF (12.50±0.16 pmol per well).

Effect of methylene blue on platelet adhesion

PAF induced platelet adhesion to endothelial cells when PMNs were present (Hirafuji & Shinoda, 1991a). Figure 5 demonstrates the effect of methylene blue on the platelet adhesion in the absence or presence of PMNs. In the absence of PMNs, PAF did not induce platelet adhesion to endothelial cells and suppression of platelet cyclic GMP formation by methylene blue had no effect. In contrast, in the presence of PMNs, the PMN-dependent platelet adhesion induced by PAF was dose-dependently increased by methylene blue.

Effects of cyclic nucleotides and sodium nitroprusside on platelet adhesion

Table 1 demonstrates the effects of membrane-permeable dibutyryl cyclic AMP, 8-bromo cyclic GMP, and sodium nitroprusside on PMN-dependent platelet adhesion. 8-Bromo cyclic GMP significantly inhibited the platelet adhesion in a dose-dependent manner, whereas dibutyryl cyclic AMP had no effect. Their combination had no further effect. Sodium nitroprusside also dose-dependently inhibited the platelet adhesion.

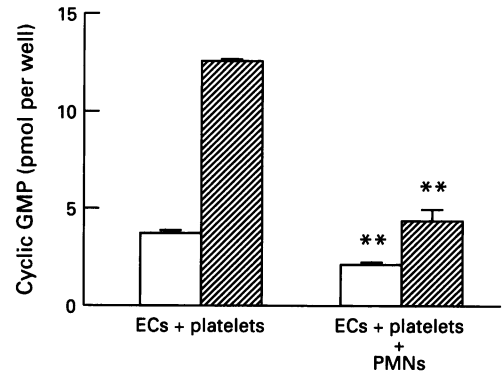

Figure 4 Cyclic GMP formation by interaction of endothelial cells, platelets and PMNs. Endothelial cells and platelets without or with PMNs were incubated in the absence (open columns) or the presence of PAF (hatched columns) for 20 min at 37°C. Mean±s.e.mean of a representative of 3 experiments performed in triplicate. $^{**}P<0.01$ vs ECs + platelets.

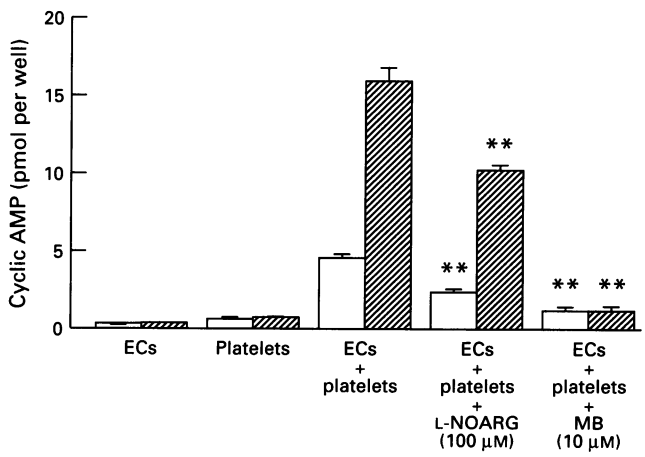

Figure 3 Effects of N^G-nitro-L-arginine (L-NOARG) and methylene blue (MB) on cyclic GMP formation by interactions of endothelial cells and platelets. Endothelial cells without or with platelets were incubated in the absence (open columns) or the presence of 10 nM PAF (hatched columns) for 20 min at 37°C. At the end of incubation, cyclic GMP was extracted and determined by enzyme-immunoassay. Mean±s.e.mean of a representative of 3 experiments performed in triplicate. $^{**}P<0.01$ vs ECs + platelets.

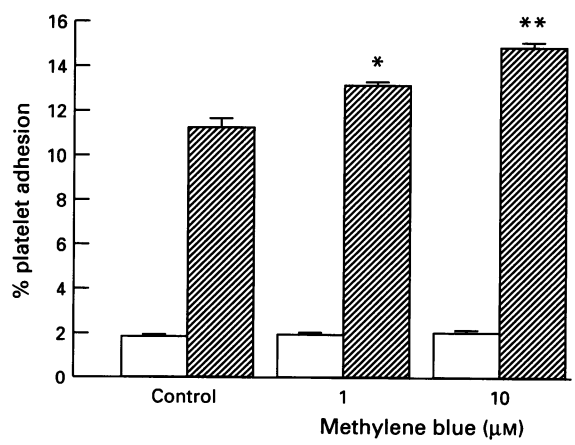

Figure 5 Effect of methylene blue on platelet adhesion to endothelial cells in the absence or the presence of PMNs. Radiolabelled platelets without (open columns) or with (hatched columns) intact PMNs were incubated for 20 min with endothelial cells in the presence of PAF (10 nM) and methylene blue at the indicated concentrations. Mean± s.e.mean of a representative of 3 experiments performed in triplicate. $^{*}P<0.05$; $^{**}P<0.01$ vs control.

Effects of cyclic nucleotides and zaprinast on platelet adhesion

Table 2 shows the effects of cyclic nucleotides and zaprinast, a cyclic GMP-specific phosphodiesterase (PDE) inhibitor, on platelet adhesion to endothelial cells induced by intact PMNs and PMN sonicates. Zaprinast alone at 10 μM slightly but significantly inhibited the intact PMN-induced platelet adhesion by 14.0%, and potentiated the inhibitory effect of 8-bromo cyclic GMP.

On the other hand, sonicates of PAF-stimulated PMNs induced mainly adhesion of platelet aggregates to aspirin- and L-NOARG-pretreated endothelial cells, as reported previously (Hirafuji & Shinoda, 1993). 8-Bromo cyclic GMP and dibutyryl cyclic AMP significantly inhibited the platelet adhesion induced by the PMN sonicates by 46.1% and 72.2%, respectively. Furthermore, zaprinast alone at 10 μM significantly inhibited the platelet adhesion induced by PMN sonicates by 55.1%. The combination of 8-bromo cyclic GMP and zaprinast had a marked inhibitory effect (77.2% inhibition) on the adhesion of platelet aggregates.

Discussion

Endothelial cells release a variety of substances, including prostacyclin and EDRF which is now believed to be identical with NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). These mediators increase platelet cyclic AMP (Moncada & Vane, 1978) and cyclic GMP levels (Mellion *et al.*, 1981), respectively. In agreement with these results, the present study demonstrated that when endothelial cells and platelets were co-incubated, the cyclic AMP level of the incubate markedly increased, and indomethacin, a cyclo-oxygenase inhibitor, significantly decreased the cyclic AMP level. Likewise, the cyclic GMP level of platelets was remarkably increased by incubation with endothelial cells. L-NOARG, a NO synthase inhibitor (Ishii *et al.*, 1990; Moore *et al.*, 1990), slightly and methylene blue, an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1981), completely inhibited the increase in cyclic GMP, suggesting that the increase is due to stimulation of platelet soluble guanylate cyclase by EDRF. PAF also caused a further increase in the cyclic GMP level in endothelial cells and platelets incubate, suggesting that PAF stimulated EDRF release from human endothelial cells.

The present study also demonstrated that addition of PMNs to endothelial cells and platelets suppressed the increases in platelet cyclic GMP levels caused by interaction with endothelial cells. This suppression was greater when PMNs were stimulated by PAF. In contrast, PMNs did not suppress the cyclic AMP level, as well as prostacyclin synthesis (Hirafuji & Shinoda, 1993). Since PAF induces platelet adhesion to endothelial cells in the presence of PMNs (Hirafuji & Shinoda, 1991a), our results indicate that the PMN-dependent platelet adhesion accompanies an impairment of platelet cyclic GMP but not cyclic AMP formation. Platelet adhesion seems to be regulated by elevation of cyclic GMP but not cyclic AMP, since EDRF, in contrast to prostacyclin, strongly inhibit platelet adhesion (Radomski *et al.*, 1987; Sneddon & Vane, 1988; Venturini *et al.*, 1992). The PAF-induced platelet adhesion is totally dependent on PMN activation, since PMNs fixed with formaldehyde and glutaraldehyde could not induce the platelet adhesion (unpublished data). In response to various stimuli, PMNs release active oxygens (Harlan, 1987), which can destroy EDRF (Gryglewski *et al.*, 1986). Since the platelet adhesion was significantly inhibited by SOD (Hirafuji & Shinoda,

Table 1 Effects of cyclic nucleotides and sodium nitroprusside on PMN-dependent platelet adhesion to endothelial cells induced by PAF

Additions		*Platelet adhesion* (% of control (*n*))
Dibutyryl cyclic AMP (Db-cAMP)	0.1 mM	98.8±0.7 (3)
	1 mM	93.4±8.6 (3)
8-Bromo cyclic GMP (Br-cGMP)	0.1 mM	87.6±1.8** (3)
	1 mM	81.4±2.9** (3)
Db-cAMP 1 mM + Br-cGMP 1 mM		79.3±2.4** (3)
Sodium nitroprusside	10 μM	89.0±3.1* (4)
	100 μM	77.0±3.5** (4)

Radio-labelled platelets and intact PMNs were incubated for 20 min with endothelial cells in the presence of PAF (10 nM) and the indicated agents. Results are expressed as a percentage of the control platelet adhesion induced by PAF. Mean±s.e.mean of 3–4 experiments performed in triplicate. *$P<0.05$, **$P<0.01$.

Table 2 Effects of cyclic nucleotides and zaprinast on platelet adhesion to endothelial cells induced by sonicates of PAF-stimulated PMNs and by intact PMNs

Additions		*Platelet adhesion induced by* *PMN soniciates*[a] (% of control)	*Intact PMNs*[b] (% of control)
8-Bromo cyclic GMP	1 mM	53.9±3.7**	81.4±2.9**
Zaprinast	10 μM	44.9±4.6**	86.0±3.6*
8-Bromo cyclic GMP + zaprinast		22.8±3.1**	71.2±4.3**
Dibutyryl cyclic AMP	1 mM	27.8±2.5**	93.4±8.6

[a]Endothelial cells were pretreated with 500 μM aspirin and 300 μM N^G-nitro-L-arginine for 15 min. After aspirating the incubation medium, [^{3}H]-adenine-labelled platelets and sonicates of PAF-stimulated PMNs were incubated for 20 min with endothelial cells in the presence of the indicated agents.
[b]Radiolabelled platelets and intact PMNs were incubated for 20 min with endothelial cells in the presence of PAF (10 nM) and the indicated agents.
Mean±s.e.mean of 3–4 experiments performed in triplicate. *$P<0.05$, **$P<0.01$.

1993), it is conceivable that decrease in platelet cyclic GMP level is due to PMN-derived active oxygens. On the other hand, a possible mechanism by which active oxygens increase platelet adhesion is suggested to be through inhibition of guanylate cyclase (Salvemini *et al.*, 1989). The possibility cannot be ruled out that PMNs interfered with platelet cyclic GMP formation by consuming EDRF as its target. Although the precise mechanism is unclear, these results suggest that disturbance in EDRF-induced platelet cyclic GMP formation by activated PMNs is involved in the mechanism of PMN-dependent platelet adhesion.

Our studies using drugs which alter platelet cyclic nucleotide levels are consistent with this hypothesis. Suppression of platelet cyclic GMP level by methylene blue dose-dependently augmented the platelet adhesion only in the presence of PMNs. Sodium nitroprusside, a NO donor, and membrane permeable 8-bromo cyclic GMP but not dibutyryl cyclic AMP significantly suppressed the PMN-dependent platelet adhesion. These inhibitory effects were further enhanced when cyclic GMP degradation was inhibited by a cyclic GMP-specific PDE inhibitor, zaprinast (Lugnier *et al.*, 1986). The inhibitory effect of these drugs may be mainly on platelets, because sodium nitroprusside does not suppress PMN functions in the concentration range used in the present study (Pieper *et al.*, 1994). The role of cyclic GMP in PMN activation and inhibition seems to be complex both enhancing and inhibiting superoxide anion formation (Ervens *et al.*, 1991), although it inhibits PMN function at high concentrations (Schröder *et al.*, 1990). Endothelial cyclic GMP does not stimulate the release of major anti-platelet substances, EDRF (Kuhn *et al.*, 1991) and prostacyclin (Doni *et al.*, 1988). Further, platelets contain mainly type III and V isozymes of PDE (Hidaka & Asano, 1976), and PMNs contain type IV PDE (Wright *et al.*, 1990), while zaprinast is a selective type I/V PDE inhibitor (Lugnier *et al.*, 1986), suggesting that the target cells of zaprinast were platelets.

Thus, the PMN-dependent platelet adhesion to endothelial cells accompanied an impairment of platelet cyclic GMP but not cyclic AMP formation, and was partially inhibited by agents which increased the platelet cyclic GMP level, while these agents markedly inhibited the adhesion of platelet aggregates induced by sonicates prepared from PAF-stimulated PMNs (Hirafuji & Shinoda, 1993). These results suggest that the interference of EDRF-induced platelet cyclic GMP formation by PMNs is involved, at least in part, in the platelet adhesion. However, the platelet adhesion could not be explained by this mechanism alone, since complete suppression of the platelet cyclic GMP level by methylene blue did not induce the platelet adhesion in the absence of PMNs. Various bioactive substances derived from activated PMNs can modulate not only platelets but also endothelial cell function (Harlan, 1987). Besides EDRF, alteration of endothelial antithrombogenic substances such as glycosaminoglycans (Bianciardi *et al.*, 1991) or 13-hydroxyoctadecadienoic acids (Buchanan *et al.*, 1991) by intercellular interactions with PMNs may trigger the platelet adhesion, which would be potentiated by suppression of the platelet cyclic GMP level.

In conclusion, the present study suggests that the impairment of EDRF-induced platelet cyclic GMP formation by PMNs is involved in part in the mechanism of PMN-dependent platelet adhesion to endothelial cells by PAF *in vitro*. The precise mechanism still remains to be clarified.

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