INFLUENCE OF ENDOTHELIN ON HUMAN PLATELET AGGREGATION AND PROSTACYCLIN GENERATION FROM HUMAN VASCULAR ENDOTHELIAL CELLS IN CULTURE

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The effects of endothelin (ET) on the function of cultured human umbilical vein endothelial cells (HUVEC) and that of human platelets were investigated with reference to endothelium-derived relaxing factor (EDRF) and PGI_2 . Considering the platelets, ET had no effect on platelet-rich plasma (PRP) aggregation, the generation of thromboxane A_2 ($[\text{TXA}_2]$) from platelets, and cytosolic free calcium ion concentration ($[\text{Ca}^{++}]_i$), cAMP content ($[\text{cAMP}]_i$) or cGMP content ($[\text{cGMP}]_i$) in platelets. In contrast, the addition of the solution in which HUVEC had been incubated with ET to PRP produced a decrease in PRP aggregation, $[\text{TXA}_2]$, and $[\text{Ca}^{++}]_i$, and an increase not only in $[\text{cAMP}]_i$ but also in $[\text{cGMP}]_i$ in platelets. In the HUVEC pretreated with acetylsalicylic acid (aspirin), this increase of $[\text{cGMP}]_i$ was not affected, but the HUVEC-mediated decrease in PRP aggregation, $[\text{TXA}_2]$, and $[\text{Ca}^{++}]_i$ induced by ET were not completely abolished. However, the pretreatment of HUVEC with a combination of aspirin and L- N^G -monomethyl arginine (LNMA) as an inhibitor of EDRF completely abolished the HUVEC-mediated decrease in PRP aggregation, $[\text{TXA}_2]$ and $[\text{Ca}^{++}]_i$ induced by ET, and also abolished the enhancement of $[\text{cGMP}]_i$ and $[\text{cAMP}]_i$ in platelets. The PGI_2 of HUVEC was enhanced by ET with no changes in $[\text{Ca}^{++}]_i$, $[\text{cAMP}]_i$ and $[\text{cGMP}]_i$. The ET-induced enhancement was remarkably attenuated by pretreating the HUVEC with aspirin, but not with LNMA.

We conclude that ET attenuates the aggregation of platelets through a decrease in $[\text{TXA}_2]$ by an increase in $[\text{cAMP}]_i$ via the increase in PGI_2 of HUVEC, and by an increase in $[\text{cGMP}]_i$ via EDRF.

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THE vascular endothelial cell produces a vasoactive peptide, endothelin, which exerts a powerful vasoconstrictive effect on smooth muscle cells¹. While the mechanism

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of its action on vascular smooth muscle cells has been investigated with reference to calcium ion kinetics, its physiological action has not been fully investigated. There are several reports on the influence of endothelin on platelet function² but its effect on platelet aggregation, and its mode of action, have not yet been clarified with reference to the interactions among the vascular endothelial cell, platelet and endothelin. We investigated the effects of endothelin-1 on cultured human umbilical vein endothelial cells (HUVEC) and platelet aggregation with respect to its mode of action.

MATERIALS AND METHODS

1) Preparation of platelet-rich plasma and measurement of platelet aggregation

Blood was drawn from fasting normal subjects who had not ingested any antiplatelet drugs such as acetylsalicylic acid (aspirin) for at least 2 weeks. Blood was mixed with 0.1 volume of 3.2% sodium citrate in polypropylene tubes and centrifuged at 250G for 10 min at room temperature to yield platelet-rich plasma (PRP). Platelet-poor plasma was obtained after centrifugation at 1500G for 15 min. Platelet-rich plasma was diluted with platelet poor-plasma and the platelet counts were adjusted to 300,000/mm³.

Aggregation was measured in a 3-channel platelet aggregometer (Rikadenki Ltd. Osaka, Japan) at 37 °C with 1 ml cuvettes filled with 500 μ l platelet suspension stirred at 1000 rpm. The baseline of the recording pen was set using the platelet suspension, and 100% aggregation was set using platelet poor plasma. The extent of aggregation was measured as the maximum increase in light transmission. The percentage of aggregation was calculated by comparison with the curve obtained in the presence of adenosine diphosphate (ADP) (final concentration 2×10^{-6} M), which was dissolved in tris buffer solution (25 mM Tris HCl, 130 mM NaCl, pH 7.4). Platelet aggregation induced by the addition of ADP was measured in the presence of 1) endothelin alone (final concentration 5×10^{-11} , 5×10^{-9} , 2×10^{-7} M); and 2) the solution in which the cultured HUVEC were incubated with endothelin in the presence or absence of aspirin, L-N^G-monomethyl arginine (LNMMA) or L-

arginine. Platelet aggregation in each experiment reached a maximum within 5 min.

2) Culture of vascular endothelial cells

Vascular endothelial cells were cultured by the method previously described³ The primary cultured cells which formed confluent monolayers in 35 mm in diameter wells were used in the following experiments. There were 2×10^5 HUVEC per well. The cells were identified as vascular endothelial cells by the detection of Weibel-Palade body on electron microscopy⁴ and by the release of angiotensin I-converting enzyme.

a) Preparation of the test solution in which HUVEC were incubated to investigate effect for platelet aggregation induced by ADP

After decanting the primary culture medium of HUVEC the cells were incubated at 37 °C with 500 μ l of the conditioned buffer solution (buffer A: 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 5 mM glucose, 1 mM MgCl₂, 10 mM HEPES, pH 7.4) containing several test reagents for 15 min, and 0.1 volume of an aliquot was added to the test tube to be used in the aggregometer. HUVEC were pretreated with aspirin, LNMMA or L-arginine, by incubation with aspirin (10^{-4} M), LNMMA (3×10^{-4} M) or L-arginine (10^{-3} M) in culture medium for 60 min. After the HUVEC were washed twice with Dulbecco's phosphate buffer solution (137 mM NaCl, 2.7 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, pH 7.4), they were further incubated at 37 °C with 500 μ l of buffer A containing several test reagents for 15 min, and a volume of 0.1 of an aliquot was added to the test tube to be used in the aggregometer.

3) Assay of cyclic AMP and GMP content in platelets and HUVEC subjected to experiments

The concentration of cyclic AMP and GMP ([cAMP]_i and [cGMP]_i) in the platelets or HUVEC was measured by the modified method of Beretz et al⁵ In brief, 300 μ l of platelet-rich plasma was first incubated with ADP (final concentration 2×10^{-6} M) or other agents for 5 min. The HUVEC was incubated in buffer A with or without endothelin (5×10^{-9} M) for 15 min. After the

TABLE I

	Platelet aggregation (%)	TXB ₂ (ng/ml/3×10 ⁸ platelets)	cAMP (pmol/3×10 ⁸ platelets)	cGMP (pmol/3×10 ⁸ platelets)	[Ca ⁺⁺] _i (nM)
Tris buffer solution	0				118.9±3.2
ADP (2×10 ⁻⁶ M)	100	478.1±44.0	45.5±2.8	40.2±3.6	214.7±12.0
ADP+ET (5×10 ⁻¹¹ M)	100.2±3.2	490.5±45.6	47.4±2.5	42.0±3.9	
ADP+ET (5×10 ⁻⁹ M)	99.8±4.1	485.1±43.1	45.3±3.0	44.9±3.5	210.1±12.7
ADP+ET (2×10 ⁻⁷ M)	100.5±3.3	483.4±43.4	47.3±3.3	42.6±3.7	217.4±13.1

(Mean±SEM)

Direct effect of endothelin-1 (ET; final concentration 5×10⁻¹¹, 5×10⁻⁹, 2×10⁻⁷ M) on platelet function (adenosine diphosphate (ADP)-induced platelet aggregation, thromboxane A₂ (TXA₂) concentration, cAMP concentration and cytosolic free Ca⁺⁺ concentration ([Ca⁺⁺]_i)).

Data are presented as mean±SEM.

TABLE II

	platelet aggregation
ADP	100
Buffer A without ET	100
#ET	
(5×10 ⁻¹³ M)	78.9±4.7**
(5×10 ⁻¹¹ M)	71.3±3.5**
(5×10 ⁻⁹ M)	57.1±5.0**
(2×10 ⁻⁷ M)	58.1±5.2**

ADP-induced platelet aggregation by addition of the medium in which human umbilical vein endothelial cells had been incubated with endothelin-1 (ET: 5×10⁻¹³, 5×10⁻¹¹, 5×10⁻⁹, 2×10⁻⁹, 2×10⁻⁷ M).

Data are presented as % aggregation for ADP-induced platelet aggregation and mean±SEM. (n=10, *, ** represents p<0.05, p<0.01 (ADP vs ET) respectively) #: concentration of ET incubated with HUVEC.

reaction was stopped by adding cold trichloroacetic acid (TCA) to achieve a final concentration of 5%, the sample was sonicated using an ultrasonic sonicator in an ice bath and centrifuged (1500G) at 4 °C for 10 min. The supernatant was obtained and TCA in the supernatant was then extracted twice with 3 volumes of water-saturated ether. The cyclic nucleotides in 100 µl of the above-treated

supernatant were then assayed using [¹²⁵I] cyclic AMP and GMP radioimmunoassay (RIA) kits (Yamasa Co. Ltd., Choshi, Japan). The assay values were estimated to be the intracellular content of the cyclic nucleotides.

4) Assay of thromboxane A₂ concentration in platelets

The concentration of thromboxane A₂ in the platelets was measured as the concentration of thromboxane B₂ by the modified method of Siess et al⁶. In brief, at the end of the aggregation study, 200 µl of platelet-rich plasma which was incubated with ADP (final concentration 2×10⁻⁶ M) or other agent for 5 min was acidified to pH 3.5 by 1N-HCl and stirred at 37 °C before the sample was transferred to acetone at -20 °C. After centrifugation at 2000G for 15 min the supernatant was extracted twice with 1 ml of ethylacetate, then evaporated to dryness in a vacuum. The sample was stored in ethanol at -20 °C. The concentration of thromboxane A₂ in 100 µl of the sample was measured as thromboxane B₂ using a [³H]thromboxane RIA kit (New England Nuclear, Boston, USA).

5) Assay of prostacyclin concentration

HUVEC were incubated with 500 µl of Buffer A containing several test reagents in

TABLE III

	TXA ₂ -generation from platelet (ng/ml/3×10 ⁸ platelets)
Endothelial cell (−) Buffer A	485.1±43.1
Endothelial cell (+) Buffer A	343.6±29.9**
Pretreatment with ASA	487.7±40.4
Pretreatment with ET	273.6±29.3**
Pretreatment with ASA & ET	436.5±40.2**
Pretreatment with ASA, LNMMA & ET	445.0±34.6*
Pretreatment with ASA, LNMMA, L-arginine & ET	475.7±32.9

Effect of the solution in which HUVEC were incubated with or without acetylsalicylic acid (ASA; 10^{−4} M), L-N^G-monomethyl arginine (LNMMA; 3×10^{−4} M), L-arginine (1×10^{−3} M) or endothelin-1 (ET; 5×10^{−9} M) on the generation of platelet thromboxane A₂ (TXA₂).

Data are presented as mean±SEM. (n=10; *, p<0.05, **: p<0.01 vs buffer A without endothelial cell)

TABLE IV

	cyclic AMP (pmole/3×10 ⁸ platelets)	cyclic GMP (pmole/3×10 ⁸ platelets)
Endothelial cell (−) Buffer A	45.2±3.0	40.3±2.8
Endothelial cell (+) Buffer A	56.2±4.1**	42.3±3.0
Pretreatment with ASA	46.4±2.5	39.1±2.4
Pretreatment with ET	63.3±4.8**	57.4±3.4**
Pretreatment with ASA & ET	47.2±1.7	56.0±3.4**
Pretreatment with ASA, LNMMA & ET	46.7±1.7	43.5±2.6
Pretreatment with ASA, LNMMA, L-arginine & ET	47.0±1.8	55.8±2.4**

Effect of the solution in which HUVEC were incubated with or without acetylsalicylic acid (ASA; 10^{−4} M), L-N^G-monomethyl arginine (LNMMA; 3×10^{−4} M), L-arginine (1×10^{−3} M) or endothelin-1 (ET; 5×10^{−9} M) on the platelet cyclic AMP and cyclic GMP generation.

Data are presented as mean±SEM. (n=10; *, ** represents p<0.05, p<0.01 vs buffer A without endothelial cell respectively)

37 °C for 15 min, and an aliquot was subjected to the assay of prostacyclin concentration. Prostaglandins mainly generated from HUVEC were confirmed to be prostacyclin by thin-layer chromatography from a preliminary experiment with [1-¹⁴C]-arachidonic acid. The prostacyclin released from HUVEC was measured as its stable metabolite, 6-keto prostaglandin F_{1α}, using [³H] 6-keto prostaglandin F_{1α} RIA kit (New England Nuclear, Boston, USA).

6) Measurement of cytosolic free calcium ion concentration

The cytosolic free calcium ion concentration was measured by the modified method of Grynkiewicz⁷. In brief, the endothelial cells were scraped from the dishes with a rubber spatula and collected in centrifugation tubes. The cells were then rinsed with 10 ml of buffer A without calcium ions containing 1.0% bovine serum albumin. After the cells were resuspended in 3 ml of buffer A containing bovine serum albumin they were incubated with 3.2 μM fura-2/AM at 37 °C for 45 min and then diluted to 20 ml with the buffer A containing bovine serum albumin. Following resuspension in 1.0% bovine

TABLE V

	platelet aggregation
Endothelial cell (–) Buffer A	100
Endothelial cell (+) Buffer A	78.9 ± 4.7**
Pretreatment with ASA	99.6 ± 0.3
Pretreatment with ET	57.1 ± 1.0**
Pretreatment with ASA & ET	94.5 ± 0.9**
Pretreatment with ASA, LNMMA & ET	99.8 ± 0.4
Pretreatment with ASA, LNMMA, L-arginine & ET	93.5 ± 1.1**

Effects of the solution in which HUVEC were incubated with or without endothelin-1 (ET; 5×10^{-9} M), acetylsalicylic acid (ASA; 10^{-3} M) on the platelet aggregation.

Data are presented as % aggregation for ADP-induced platelet aggregation and mean ± SEM. (n=10; *, ** represents $p < 0.05$, $p < 0.01$ vs buffer A without endothelial cell respectively)

TABLE VI

	$[Ca^{++}]_i$ of the platelet (nM)
Endothelial cell (–) tris buffer solution	118.9 ± 3.2
ADP	214.7 ± 12.0**
Endothelial cell (+) Pretreatment with ET	155.1 ± 9.5**
Pretreatment with ASA & ET	181.1 ± 5.4**
Pretreatment with ASA, LNMMA & ET	197.8 ± 17.1**
Pretreatment with ASA, LNMMA, L-arginine & ET	179.7 ± 5.6**

Effects of the solution in which HUVEC were incubated with or without acetylsalicylic acid (ASA; 10^{-4} M), L-NG-monomethyl arginine (LNMMA; 3×10^{-4} M), L-arginine (1×10^{-3} M) or endothelin-1 (ET; 5×10^{-9} M) on the platelet cytosolic free calcium concentration.

Data are presented as mean ± SEM. (n=10; *, ** represents $p < 0.05$, $p < 0.01$ vs tris buffer solution without endothelial cell respectively)

serum albumin solution (cell count 10^6 /ml) they were centrifuged at 250G for 10 min. Fluorescence signals from the endothelial cell suspensions in the supernatant were recorded in UV-compatible cuvettes utilizing a calcium ion analyzer CAF-100 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). At first calcium chloride (final concentration 1.8 mM) was added to the cell suspension, and then when the fluorescence signals reached a plateau, vasoactive substances such as endothelin (final concentration 5×10^{-9} M) or thrombin (final concentration 10 U/ml) were added to the cell suspension.

The cytosolic free calcium ion concentration of the platelets was also measured by

the method described. In brief, platelets were rinsed with 10 ml of buffer A without calcium ions containing 1.0% bovine serum albumin. After the platelets were resuspended in 3 ml of the above solution, they were incubated with $3.2 \mu\text{M}$ fura-2/AM at 37 °C for 45 min and then diluted to 20 ml with the above solution. Following resuspension in 1.0% bovine serum albumin (platelet count 10^6 /ml), they were centrifuged at 250G for 10 min. Fluorescence signals from the platelet suspensions were recorded in UV-compatible cuvettes utilizing a calcium ion analyzer CAF-100. Calibration was done as follows: the maximum fluorescence was obtained by the disruption of HUVEC or the

Fig.1. Effect of endothelin-1 (ET; 5×10^{-13} , 5×10^{-11} , 5×10^{-9} , 2×10^{-7} M) on the generation of PGI₂ by human umbilical vein endothelial cells; Effect of pretreatment with acetylsalicylic acid (ASA, 10^{-4} M) on endothelin-1 (ET, 5×10^{-9} M) induced PGI₂ release from human umbilical vein endothelial cells. Data are presented as mean $\pm$ SEM. (n=8; *, ** represents $p < 0.01$, $p < 0.001$ respectively)

Fig.2. Effect of endothelin-1 (ET; 5×10^{-9} M) or thrombin (Th; 10 U/ml) on cytosolic free Ca⁺⁺ concentration of human umbilical vein endothelial cells.

platelets with 0.1% Triton X100, and the minimum fluorescence was obtained by the subsequent addition of 1 mM EGTA, pH 8.0.

7) Reagents

The following reagents were used in this experiment: bovine serum albumin (Sigma fraction V, St. Louis, USA), porcine endothelin-1 (Peptide Institute Inc., Osaka, Japan), fura-2 acetomethoxy pentaester (fura-2/AM, Calbiochem, La Jolla, USA), adenosine diphosphate (Sigma Chemical Co., St. Louis, USA), L-N^G-monomethyl arginine (LNMA, gift from I. Sakuma

[Hokkaido University, Japan]), L-arginine (Nacalai Tesque, Inc., Kyoto, Japan), acetylsalicylic acid (Nacalai Tesque, Inc., Kyoto, Japan), and human thrombin (Green Cross Corporation, Osaka, Japan).

6) Statistical analysis

Data (expressed as mean $\pm$ SEM) were compared using analysis of variance. For F-ratios significant at the 5% level or less, Duncan's multiple range test was applied to test for differences between any two groups. Differences at the 5% level or less ($p < 0.05$) were considered statistically significant. Percentage changes, for which a normal dis-

TABLE VII

	cyclic AMP (fmole/ 2×10^5 cells)	cyclic GMP (fmole/ 2×10^5 cells)
Buffer A	38.2 ± 2.2	65.4 ± 4.9
ET	37.1 ± 2.0	66.9 ± 3.7

Effect of endothelin-1 (ET; 5×10^{-9} M) on the cyclic AMP and cyclic GMP generation of human umbilical vein endothelial cells.

Data are presented as mean $\pm$ SEM.

tribution cannot be assumed, were compared using the Kruskal-Wallis non-parametric method for analysis of variance. Whenever the chi-square test results were significant, the Mann-Whitney test was used to determine the significance of the differences between pairs of means.

RESULTS

1) Effect of endothelin on platelet function

The addition of endothelin (5×10^{-11} , 5×10^{-9} , 2×10^{-7} M) to PRP had no effect on the ADP-induced platelet rich plasma aggregation, the concentration of thromboxane A_2 , $[cAMP]_i$, $[cGMP]_i$ or the $[Ca^{++}]_i$ of the platelets (Table I).

2) Function of platelets by adding solution in which HUVEC were incubated with endothelin

The addition of the solution in which HUVEC were incubated with buffer A to platelet-rich plasma caused a decrease of both platelet aggregability and concentration of thromboxane A_2 in the platelets. In this experiment, $[cAMP]_i$ of the platelets increased with no change in $[cGMP]_i$ (Table III–V). These changes in platelet function were prevented by pretreatment with aspirin (Tables III–VI).

The addition of the solution in which HUVEC were incubated with endothelin to platelet-rich plasma caused a decrease in the concentration of thromboxane A_2 (Table III), and $[Ca^{++}]_i$ in the platelets (Table VI), and an increase in $[cAMP]_i$ and $[cGMP]_i$ (Table IV), and a decrease in platelet aggregability, all in a dose-dependent manner. The decrease reached a plateau at 5×10^{-9} M of endothelin (Table II). The endothelin

induced decrease in platelet function recovered partially in the presence of aspirin (Table III–VI). Namely, when HUVEC were pretreated with aspirin, the addition of the solution in which HUVEC were incubated with endothelin (5×10^{-9} M) to platelet rich plasma decreased the endothelin-induced increase of $[cAMP]_i$, but had no effect of the endothelin-induced increase of $[cGMP]_i$; the endothelin-induced decrease of platelet aggregability, the concentration of thromboxane A_2 , and $[Ca^{++}]_i$ in the platelets was not completely abolished (Tables III–VI). Furthermore the combined pretreatment of HUVEC with aspirin and L-NG-monomethyl arginine (LNMMA) (3×10^{-4} M) completely abolished the HUVEC-mediated decrease of platelet-rich plasma aggregation by endothelin, thromboxane A_2 generation and $[Ca^{++}]_i$. It also abolished the enhancement of $[cGMP]_i$ as well as that of $[cAMP]_i$ in the platelets, while the inhibitory effect of LNMMA was abolished by pretreatment with L-arginine (1×10^{-3} M) (Table III–VI).

3) Effects of endothelin on the generation of PGI_2 by HUVEC

The generation of PGI_2 from HUVEC was enhanced by endothelin (5×10^{-11} , 5×10^{-9} , 2×10^{-7} M) in a dose-dependent manner. This enhancement reached a plateau at 5×10^{-9} M (Fig. 1). However, the addition of endothelin (5×10^{-9} M) to the cell suspension of HUVEC had no effect on $[Ca^{++}]_i$ (Fig. 2), $[cAMP]_i$ and $[cGMP]_i$ (Table VII). When the HUVEC were pretreated with aspirin, the basal and endothelin-induced enhancement of PGI_2 generation decreased remarkably (Fig. 1).

DISCUSSION

Vascular endothelial cells have recently been shown to display an antithrombotic action via the generation of PGI_2 ,⁸ tissue plasminogen activator,⁹ or thrombomodulin,¹⁰ and to regulated the contraction and relaxation of smooth muscle cells via the generation and release of such vasoactive substances as endothelium-derived relaxing factor (EDRF),¹¹ PGI_2 and endothelin. There are numerous reports concerning the mechanism of their generation, release and mode of

action. Endothelin, a novel 21-amino acid peptide purified from the culture medium of porcine aortic endothelial cells, has been recognized to be a highly potent vasoconstrictor; its physiological role has been investigated.¹

The effect of endothelin on vascular smooth muscle cells include the activation of voltage-dependent Ca^{++} channel in smooth muscle cells,¹² an activation of phospholipase C^{13} and phospholipase A_2 ,¹⁴ and calcium ion mobilization from the storage site via an enhancement of IP_3 generation.¹⁵ Since few reports have investigated the influence of endothelin on the platelet and the vascular endothelial cell, we studied the effects of endothelin on HUVEC and human platelet aggregation *in vitro*.

In this study the addition of endothelin alone to human platelets had no effect on ADP-induced platelet aggregation. This finding was supported by the observation in which the direct addition of endothelin to platelet rich plasma had no effect on the concentration of thromboxane A_2 , $[\text{cAMP}]_i$ or $[\text{Ca}^{++}]_i$ of the platelet.

On the other hand, from the observation that the addition of the solution in which HUVEC were incubated with buffer A decreased ADP-induced platelet aggregation, but that its inhibition was remarkably attenuated when HUVEC were pretreated with aspirin, we inferred that the inhibition of platelet aggregation was caused by prostanoids such as the PGI_2 derived from vascular endothelial cells.

Thiemermann et al.¹⁶ reported that endothelin inhibited the ADP-induced platelet aggregation in the anesthetized rabbit, and that indomethacin abolished the endothelin-induced inhibition of platelet aggregation. Those investigators concluded that endothelin inhibited the function of platelets *in vivo* due to the release of PGI_2 or PGD_2 . While that conclusion was obtained from a study of the inhibitory effects of indomethacin without the direct measurement of prostaglandins, our experimental results support those findings.

It is well known that vascular endothelial cells produce PGI_2 and inhibit platelet aggregation. The mechanism of this action is considered due to an increase in cAMP in the platelets induced by PGI_2 , accompanied

by a decrease in thromboxane A_2 generation.^{17,18} We previously reported the inhibitory mechanism of platelet aggregation by human vascular endothelial cells.³ Pretreatment of vascular endothelial cells with endothelin indicated the above-mentioned mechanisms. The endothelin-induced inhibition of platelet aggregation is thought to be developed by the increased cAMP concentration in the platelet via the enhancement of prostanoids such as PGI_2 released from the endothelial cells by endothelin. Since in the pretreatment study of HUVEC with aspirin the addition of the solution in which HUVEC were incubated with endothelin to PRP decreased the endothelin-induced increase of $[\text{cAMP}]_i$, but had no effect on the endothelin-induced increase of $[\text{cGMP}]_i$, and since the endothelin-induced decrease of platelets aggregability and the concentration of thromboxane A_2 and $[\text{Ca}^{++}]_i$ in the platelets were not completely abolished, it seemed that an enhancement by such other substances as EDRF was involved in the inhibitory effect on platelet aggregation. We therefore investigated the implied effect of EDRF on this mechanism, utilizing LNMMA as an inhibitor of EDRF.¹⁹ The combined pretreatment of HUVEC with aspirin and LNMMA completely abolished the HUVEC-mediated, endothelin-induced decrease of platelet-rich plasma aggregation, while the inhibitory effect of LNMMA disappeared with pretreatment of L-arginine. It was thus suggested that endothelin inhibited the aggregation of platelets not only via PGI_2 generation, but also via the stimulation of EDRF release.

Simultaneously, it was shown by radio immunoassay that endothelin increased PGI_2 generation in human vascular endothelial cells. There are previous reports concerning the PGI_2 enhancement with endothelin. For instance, de Nucci et al.²⁰ reported that endothelin released PGI_2 and thromboxane A_2 in pig or rat isolated lungs. Rae et al.²¹ reported that endothelin enhanced PGI_2 and PGE_2 generation, using bioassay or RIA methods in the isolated kidney and spleen of the rabbit. Our results support those reports. Our study revealed that a lower concentration of endothelin (less than 5×10^{-9} M) enhanced PGI_2 generation. The amount reached a plateau at concentrations exceed-

ing 5×10^{-9} M endothelin, and endothelin enhanced PGI₂ generation of human vascular endothelial cells without a change in the $[Ca^{++}]_i$.

In a previous study of HUVEC we reported the mechanism of PGI₂ generation and release with reference to Ca^{++} , Na^{+} kinetics and cyclic nucleotides²² those experimental results indicate that $[Ca^{++}]_i$ does not contribute to the endothelin-induced PGI₂ enhancement of vascular endothelial cells. This enhancement may be due to such mechanisms as; 1) an influence on the kinetics of cyclic nucleotides; 2) an influence on Na^{+} kinetics such as Na^{+} - K^{+} ATPase or Na^{+} - Ca^{++} exchange system; or 3) a mechanical stimulation of the endothelial cell membrane.

In conclusion, endothelin synthesized in the vascular endothelial cells is thought to 1) enhance PGI₂ generation in endothelial cells, despite a lack of any direct influence on platelet aggregation; and 2) to inhibit platelet aggregation through an increased cAMP in the platelet, and even more so, through a decrease in thromboxane A₂ generation. Simultaneously, endothelin is thought to enhance the release of EDRF from endothelial cells, and to inhibit platelet aggregation through an increase in cGMP in the platelet and more so through the decrease in thromboxane A₂ generation.

It is unclear whether the endothelin-induced PGI₂ generation and EDRF release may act as a feedback mechanism for endothelin-induced vasoconstriction, because the vasoconstrictive activity of endothelin lasts for a longer period while the activity of PGI₂ or EDRF lasts for a short period. Further investigation is required to clarify the complex interactions discussed.

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