$GABA_A$ receptors although it is necessary to investigate further the molecular basis of the changed response to barbiturates before the hypothesis can be considered fully established.

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Potentiating effects of endothelin on platelet activation induced by epinephrine and ADP

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Endothelin (ET) is a potent vasoconstrictive peptide that has been recently characterized from the supernatant fraction of cultured vascular endothelial cells. It is the only endothelial cell-derived vasoconstrictive substance convincingly identified to date [1]. This property has been demonstrated in a variety of species *in vitro* and *in vivo*.

It would aid hemostatics if ET potentiates platelet function in cooperation with its vasoconstrictive property at the site of endothelial injury. This assumption led us to investigate the potentiating effect of ET on platelet activation induced by epinephrine and ADP.

Materials and Methods

Endothelin-1 (human) was obtained from Peptide Institute Inc. (Osaka, Japan). It was chemically synthesized, and 99% pure, determined with HPLC systems. It contained no other proaggregatory agents. STA_2, a stable thromboxane A_2 analogue, was a generous gift from Ono Pharmaceutical Corp. (Osaka, Japan). Aequorin, 2-7,bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), $DiSC_3(5)$ were obtained from Baxter Travenol (Tokyo, Japan), Dojin Chemistry (Kumamoto, Japan), and Molecular Probes, Inc. (Junction City, OR, U.S.A.), respectively.

Citrate-anticoagulated blood was obtained from healthy human donors who denied having taken any drugs for 14 days preceding the experiment. The blood was centrifuged at 60 *g* for 10 min to obtain platelet-rich plasma (PRP). PRP was adjusted to a concentration of 3×10^8 cells/mL with the corresponding platelet-poor plasma. Five hundred μL of PRP was used to measure aggregation in a Nikou Bioscience Hematracer IV (Tokyo, Japan). Maximal decrease in optical density was defined as maximal aggregation rate (MAR).

In every experiment, subthreshold doses of epinephrine and ADP were determined, which induced a decrease in MAR by less than 40%. Various doses of ET were added to the PRP and the mixture was incubated for 3–5 min at 37°. After the addition of epinephrine or ADP at subthreshold dose, platelet aggregation was measured for 10 min. ET potentiation was determined as "positive" when MAR

was increased by more than 40% above the value without preincubation of ET.

The changes in cytoplasmic pH (pHi) were measured with a pH-sensitive probe, BCECF [2]. The membrane potential changes were measured with $DiSC_3(5)$ with a Hitachi F-4010 Fluorescence Spectrophotometer [3]. Measurement of intracellular Ca^{2+} $[Ca^{2+}]_i$ was performed with aequorin-loaded platelets essentially as described by Johnson *et al.* [4] with a Platelet Ionized Calcium Aggregometer (Chrono-log, PA, U.S.A.).

Results and Discussion

ET at all concentrations tested induced no observable platelet aggregation. The potentiating effects of ET were tested on various concentrations of agonists (Fig. 1). The potentiating effects of ET was demonstrative with the subthreshold doses of epinephrine, while it was hardly observed with a full dose or with a very low dose of agonists. Thus, we chose to evaluate the potentiating effects of ET on subthreshold doses of agonists. ET potentiated epinephrine-induced platelet aggregation in 52.2% of all the individuals (12/23), and in 60.5% of all the measurements (23/38). Correlation between the original MAR values and the corresponding ET-potentiated MAR is shown in Fig. 2. The plotted data appear to consist of two distinct groups, one with positive ET potentiation, and the other not affected by ET. These findings suggest that there are individual differences in the response of platelets to ET potentiation.

One explanation for the individual differences in responsiveness may be that neutral peptidase present in plasma degradates ET in individuals who do not respond to ET. However, E64C, which is a neutral peptidase inhibitor [5], did not bring about the potentiating effect of ET on non-responders (data not shown). Furthermore, ET potentiation of non-responders was not seen at any higher concentrations of ET (2–10 μM). Thus, peptidase-induced breakdown of ET appears not to be the cause for non-responsiveness.

Optimal concentrations of ET for potentiation were diverse; ET potentiation for epinephrine-induced aggregation was observed as low as 0.2 μM ET in eight measurements, while maximal potentiation were observed at 1 μM ET in the majority of the measurements (20/23). The representative traces of aggregation are shown in Fig. 3. The individuals with positive ET potentiation of epinephrine-induced aggregation were further examined for a second study. ET potentiation of epinephrine-induced aggregation was invariably observed in 4/6 of these individuals, while there were occasionally slight differences in the optimal concentration of ET.

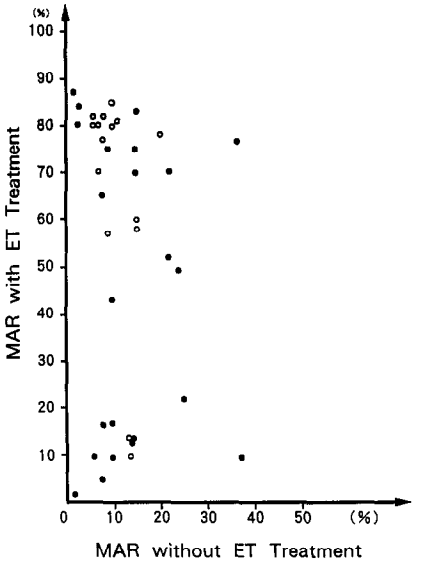

Fig. 2. Correlation between endothelin-potentiated aggregation and original aggregation. The subthreshold dose of epinephrine judged by the criteria described in Materials and Methods was added to PRP with or without ET pretreatment. MAR with ET pretreatment was plotted against original aggregation without ET. (Abscissa, MAR without ET; Ordinate, MAR with ET pretreatment.) Closed circles represent male cases and open circles represent female cases.

Furthermore, we evaluated ET potentiation of aggregation induced by other agents. ET potentiation of ADP-induced aggregation was observed in 2/24 individuals (8.3%) judged by the criteria described in Materials and Methods. In six individuals, the aggregation patterns

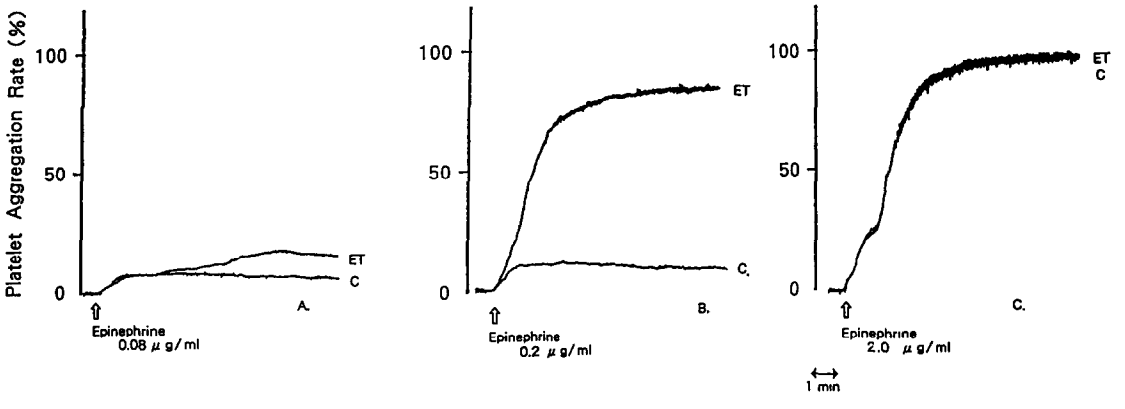

Fig. 1. Effect of endothelin pretreatment upon platelet aggregation induced by various concentrations of epinephrine. Various concentrations of epinephrine were added to PRP with or without pretreatment of 1 μM ET at the time indicated as an open arrow, and the platelet aggregation was measured with a NBS Hematracer IV. The traces with ET pretreatment are indicated by "ET", the control traces, as "C". Panels A, B and C represent the aggregation traces induced by a very low concentration, by subthreshold dose, and by a full dose of epinephrine, respectively. Each trace is representative of at least four measurements.

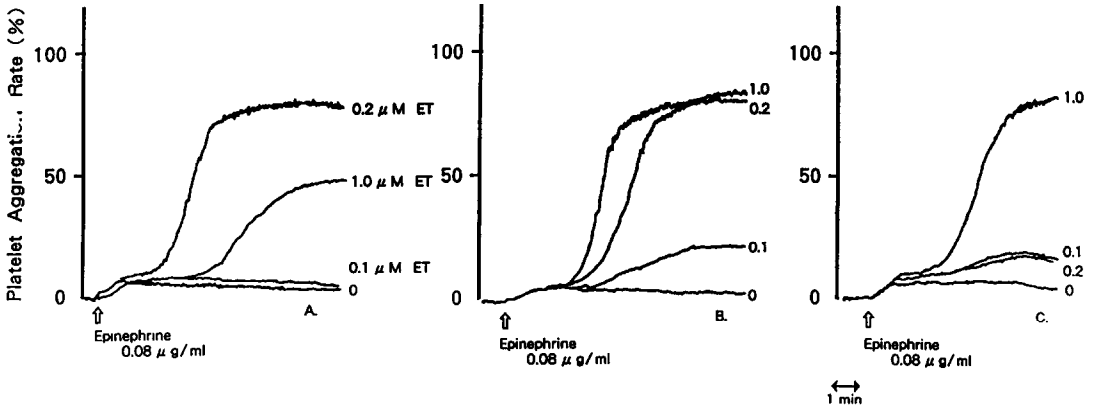

Fig. 3. Effect of endothelin pretreatment upon epinephrine-induced aggregation. The subthreshold dose of epinephrine was added to PRP with pretreatment of 0–1.0 μM ET at the time indicated by an open arrow, and the platelet aggregation was measured with a NBS Hematracer IV. Each trace is representative of 3 experiments in panel A, 5 in panel B, 15 in panel C.

showed the second wave of aggregation upon ET treatment, although the increase in MAR did not fully meet the criteria of ET potentiation described in Materials and Methods. These may be interpreted as positive cases of ET potentiation.

The effect of ET was observed neither on thrombin-induced nor on STA_2-induced platelet aggregation. Thrombin and STA_2 have been known to be potent activators of platelet activation, and the signal transduction pathways involved may be multiple. It may be that the signal transduction pathway used for ET potentiation is already involved in platelet activation induced by these potent agonists, and therefore, ET lacks its potentiating effect in these cases.

In order to elucidate the mechanisms of the ET potentiation, we measured $[Ca^{2+}]_i$ changes, pHi changes, and membrane potential changes induced by epinephrine. ET itself at all concentrations induced no significant change. ET also had not any significant potentiating effects in epinephrine-induced changes on these factors.

Tierman *et al.* [6] presented evidence that porcine ET given to anesthetized rabbits inhibits platelet aggregation probably through the release of PGI_2 and that the early anti-aggregatory effect of ET *in vivo* is most likely mediated via the release of PGI_2. The inhibitory effect of ET is also brought about by the release of EDRF (endothelium-derived relaxing factor) induced by ET [7] since EDRF also inhibits platelet function [8]. However, to the best of our knowledge, there has been no *in vitro* experiment which demonstrates a direct effect of ET on platelet function.

In summary, we report ET potentiation of epinephrine-induced platelet aggregation in approximately a half of all the individuals. Possible ET potentiation was also observed with ADP-induced aggregation in 8/24 individuals. ET, however, had no significant potentiation effects in epinephrine-induced changes on the factors such as $[Ca^{2+}]_i$, pHi, and the membrane potential changes. Although the biochemical basis underlying this action remains unknown, our findings suggest that ET modifies platelet activation induced by physiological agonists.

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