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INVOLVEMENT OF THE MICROTUBULAR SYSTEM IN THE ENDOTHELIN-1 SECRETION FROM PORCINE AORTIC ENDOTHELIAL CELLS

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Abstract—The effects of certain microtubule-disrupting agents on endothelin-1 (ET-1) secretion from porcine aortic endothelial cells were studied. When endothelial cells were treated with thrombin (1 unit/mL), a significant increase in ET-1 secretion was detected in the incubation medium, while ET-1 secretion in the medium was diminished when the cells were treated simultaneously with either colchicine or vinblastine (10^{-8}–10^{-6} M). In such cases, however, the ET-1 content detected in the cells increased dose-dependently in accordance with the concentrations of the microtubule-disrupting agents. The intracellular accumulation of ET-1 was observed both in mitochondrial and microsomal fractions. On the other hand, thrombin produced a significant increase in polymerized tubulin content without affecting the total tubulin content. A thrombin-induced increase in the intracellular Ca^{2+} concentration of endothelial cells was inhibited by treatment with either colchicine or vinblastine. These results seem to indicate that the microtubular system may play an important role in ET-1 secretion from endothelial cells.

Endothelin (ET-1) is a novel 21 amino acid vasoactive peptide containing two disulfide bonds, which was isolated originally from the culture supernatant of porcine aortic endothelial cells [1]. It has been shown that ET-1 produces a potent and sustained vasoconstriction both *in vivo* and *in vitro* in a wide variety of species [2–4]. The early signaling events of ET-1-induced vasoconstriction are associated, at least in part, with the mobilization of Ca^{2+} from internal pools and the hydrolysis of phosphatidylinositol [5, 6]. So far, it is known that the secretion of ET-1 from endothelial cells is stimulated by several substances, such as thrombin, transforming growth factor β, arg-vasopressin and angiotensin II; also, intracellular Ca^{2+} and protein kinase C seem to play an important role in the process leading to ET-1 secretion [7–9]. Since only a few secretory granules were observed in the endothelial cells, even with electron microscopy, and the level of preproendothelin mRNA in the cells increased after exposure to an appropriate stimulus, the secretion of ET-1 would seem to be regulated mainly at the level of mRNA transcription but is not dependent on the release process of the endothelin-containing granules [1]. Meanwhile, it has been mentioned that in various secretory cells the microtubular–microfilamentous system plays an essential role in the intracellular transport of the secretory granules and Golgi-derived vesicles [10]. This may be applicable to ET-1 secretion. The synthesized ET-1 is probably transferred through the microtubular system from the ribosome to the cell surface. Thus, the present study was undertaken to investigate the role of the microtubular system in the process leading to the ET-1 secretion elicited by thrombin in cultured porcine aortic endothelial cells.

MATERIALS AND METHODS

Cell culture. The endothelial cells were harvested by scraping the intimal surface of porcine aorta with a scalpel blade and were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% fetal calf serum, 100 units/mL penicillin (Meiji) and 100 μg/mL streptomycin (Meiji) in a humidified atmosphere of 5% CO_2/95% air. Endothelial cells were identified by a typical "cobblestone"-like appearance under a phase contrast microscope (TMD, Nikon) and were simultaneously confirmed by means of immunofluorescence observation of "factor VIII antigen" [11]. After the cells had grown to confluence in 24-well plates (Coning), they were washed twice with 300 μL of serum-free DMEM and incubated in 300 μL of the same medium containing one of the test agents, colchicine, vinblastine, cycloheximide and actinomycin D (all from the Sigma Chemical Co., St Louis, MO, U.S.A.).

Cell fractionation. The endothelial cells, after being incubated with 10 mL of DMEM in the presence and absence of the test agents for 6 hr in 100 mm dishes, were rinsed three times with 10 mL of ice-cold phosphate buffered saline (PBS) and homogenized in 1 mL of a homogenizing buffer (250 mM sucrose, 1 mM EDTA and 5 mM HEPES, pH 7.4) using a Potter–Elvehjem homogenizer equipped with a teflon pestle. The homogenate was centrifuged at 700 *g* for 10 min to remove the nucleus fraction, which is a mixture of nuclei and cell debris. The supernatant was centrifuged at 7000 *g* for 10 min to precipitate the mitochondrial fraction containing

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the granules and lysosomes. Subsequent centrifugation of the supernatant at 105,000 *g* for 60 min was carried out to obtain the cytosol fraction as a supernatant and the microsomal fraction as a precipitate containing the microsomes and Golgi elements. The pellets were treated with 200 μL of a solubilizing buffer [consisting of 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.3% bovine serum albumin (BSA), 0.1% NaN_3 and 50 mM phosphate buffer, pH 7.4] for 1 hr and the insoluble materials were separated by centrifugation at 105,000 *g* for 30 min.

Radioimmunoassay. The ET-1 content of the culture medium and the cells were determined by radioimmunoassay (RIA) using a specific antiserum for ET-1 (Peptide Institute, Osaka, Japan) and [^{125}I]ET-1 (Amersham International, Amersham, U.K.) as a tracer. Cross-reactivity of the antibody with big ET-1 was less than 0.1%. The assay buffer for RIA was 50 mM phosphate buffer (pH 7.4) containing 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% BSA and 0.1% NaN_3. The mixture, consisting of 100 μL of the sample or standard synthetic ET-1 (Peptide Institute), 300 μL of assay buffer and 100 μL of anti-ET-1-serum (final dilution 1:10,000), was incubated for 24 hr at 4°. Then, 100 μL of [^{125}I]ET-1 (approximately 5000 cpm) were added to each tube and incubated for another 24 hr at 4°. After that, 250 μL of Amarlex-M donkey anti-rabbit serum (Amersham International) were added to each tube and incubated for 10 min at room temperature. After a magnetic separation, the amount of radioactivity in the antibody-bound fraction was counted by a γ spectrometer (ARC-1000, Aloka).

[^{3}H]*Colchicine binding.* The endothelial cells were incubated in 60 mm dishes containing 2.5 mL of DMEM in the presence and in the absence of 1 unit/mL thrombin. The measurements of colchicine binding activity of the total and polymerized tubulin were carried out in accordance with the method of Steiner and Ikeda [12]. Briefly, to measure the polymerized tubulin, the cells were rinsed three times with 4 mL of PBS and were then incubated in 1.2 mL of microtubule stabilizing buffer (1 M glycerol, 10% dimethyl sulfoxide, 0.04% nonidet P-40, 1 mM EGTA, 1 mM guanosine triphosphate, 0.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 10 mM PIPES, pH 6.8; MSB) for 30 min at 22°. The microtubules were separated by centrifugation at 95,000 *g* for 60 min at 22°. The sedimented microtubules were resuspended in 500 μL of colchicine binding buffer (200 mM NaCl, 5 mM $MgCl_2$, 1 mM guanosine triphosphate, 1 mM phenylmethylsulfonyl fluoride and phosphate buffer, pH 6.8; CBB) and were disassembled by exposure to 4° for 30 min after sonication (20 kHz, 30 sec, UR-20P, Tomy). After removing the particulate matter by centrifugation at 60,000 *g* for 30 min at 4°, the colchicine binding activity in the supernatant was measured. Samples were incubated with 10 μM [^{3}H]colchicine (300 mCi/mmol, Dupont) for 90 min at 37°. To determine nonspecific binding, duplicate blanks pretreated with 10 mM "cold" colchicine before the [^{3}H]colchicine incubation were processed with the experimental samples. Diethylaminoethyl cellulose filter discs (DE81, Whatman) were used to trap the colchicine binding protein. The radioactivity of the washed discs was measured in a liquid scintillation spectrometer (LSC-700, Aloka). To measure the total tubulin content, the cells were rinsed with PBS and suspended in 1.2 mL of ice-cold CBB. The suspension was sonicated and kept at 4° for 30 min. After centrifugation at 60,000 *g* for 30 min at 4°, the supernatant was used for the colchicine binding assay.

Measurement of intracellular Ca^{2+} *concentration.* The endothelial cells grown on a cover slip were washed with PBS and incubated with 5 μM fura 2/AM (Dojindo) dissolved in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4; BSS) at 37° for 1 hr. Thereafter, the cells were washed twice with PBS and placed in a microchamber on the stage of a fluorescence microscope (XF, Nikon) kept at 37°. The fluorescence images of fura 2-loaded endothelial cells were observed by means of a fluorescence microscope connected to a video-intensified microscopy system (ARGUS-100, Hamamatsu). The fluorescence intensity of the fura 2–Ca complex was measured at the 510 nm band using an excitation beam passed through 340 nm (F_{340}) and 360 nm (isosbestic point of fura 2; F_{360}) filters. The relative changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were expressed as the changes in the ratio of F_{340}/F_{360}, since the autofluorescence of the cell made the measurement of the exact $[Ca^{2+}]_i$ impossible.

Statistical analysis. The data were expressed as means ± SEM. Statistical estimation was conducted using variance analysis and Dunnett's test.

RESULTS

The effect of thrombin on the secretion of immunoreactive ET-1 (IR-ET-1) from porcine aortic endothelial cells is shown in Fig. 1. Under basal conditions, a gradual increase in IR-ET-1 secretion into the control medium containing only endothelial cells was observed (Fig. 1A). When the endothelial cells were treated with thrombin at a concentration of 1 unit/mL, the amount of IR-ET-1 detected in the medium increased rapidly and the difference in ET-1 secretion between non-treated and thrombin-treated cases became more and more apparent with increasing incubation time. During treatment with thrombin (0.1–10 units/mL) for 6 hr, IR-ET-1 secretion was elicited in a concentration-dependent manner, and at a concentration of 1 unit/mL, the IR-ET-1 secretion reached its maximum (Fig. 1B). In the case of basal ET-1 secretion, colchicine and vinblastine (10^{-8}–10^{-6} M) caused a moderate but dose-dependent decrease in ET-1 secretion from the endothelial cells and the percentage of inhibition elicited at a concentration of 10^{-6} M was 16.9 ± 3.5% and 17.5 ± 3.1%, respectively. When colchicine or vinblastine was added to the medium simultaneously with thrombin (1 unit/mL), at concentrations ranging from 10^{-8} to 10^{-6} M, the IR-ET-1 secretion induced by thrombin was inhibited dose-dependently (Fig. 2A). However, treatment with lumicolchicine, which was performed similarly, did not inhibit the IR-ET-

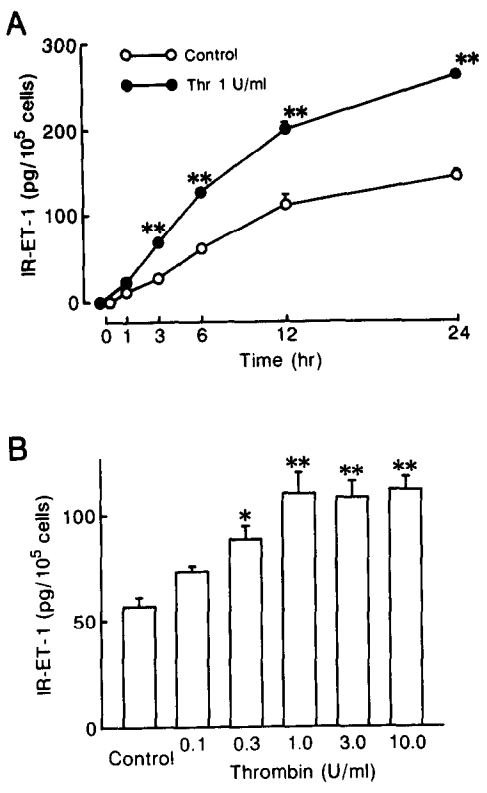

Fig. 1. Time-course of IR-ET-1 secretion (A) and concentration-dependent secretion of IR-ET-1 induced by thrombin (Thr) (B) from porcine aortic endothelial cells. (A) The endothelial cells were grown to confluence in a 24-well plate and incubated with or without thrombin at a concentration of 1 unit/mL; and (B) incubated for 6 hr at various concentrations of thrombin. Each point represents mean ± SEM of 5 experiments. *, **; Significantly different from the control group at $P < 0.05$ and $P < 0.01$, respectively.

1 secretion even at a concentration of 10^{-6} M (data not shown). By contrast, when the ET-1 content of the cells was measured under the same conditions, the amount of IR-ET-1 increased dose-dependently with increasing concentrations of colchicine or vinblastine (Fig. 2B). When the endothelial cells were stimulated by thrombin (1 unit/mL) in the presence of cycloheximide or actinomycin D, at concentrations ranging from 10^{-9} M to 10^{-7} M or from 10^{-10} M to 10^{-8} M, not only was there a decrease in the amount of ET-1 released into the medium but also in the ET-1 content of the cells (Fig. 3A and B). Notably, the amount of ET-1 released into the medium was suppressed dose-dependently and significantly. Since the localization of ET-1 in the endothelial cells has not yet been defined clearly, changes in the intracellular content of IR-ET-1 in various fractions of endothelial cells stimulated with thrombin, in the presence and absence of microtubule-disrupting agents, were investigated by means of differential centrifugation. In the case of differential fractionation, it is possible that ET-1, which was deaggregated during the cell homogenization, may bind to one or more components of organelles and this could be the reason for misinterpretation of the intracellular localization of ET-1. To investigate such a possibility, [^{125}I]ET-1 (4000 cpm/2 fmol) was added to the homogenate and fractionation was performed as described earlier. Subsequently, less than 2% of the total radioactivity was detected in each of the following: the nuclear fractions, the mitochondrial fraction and the microsomal fraction; but almost all the remaining radioactivity (more than 94%) was recovered from the cytosol fraction (data not shown). This indicates clearly that even when ET-1 is detached during cell homogenation and fractionation, it may not bind to some particular organelles. As shown in Fig. 4, a significant increase in the intracellular IR-ET-1 content was noticed in the mitochondrial and microsomal fractions obtained from cells treated in the presence of either colchicine or vinblastine. When the colchicine binding activity of the total and polymerized tubulins in the resting endothelial cells was determined, the total tubulin content of the endothelial cells was 17.7 ± 0.5 μg/10^6 cells (eight experiments), 32.2 ± 1.3% of which existed in the polymerized form. The treatment of endothelial cells with 1 unit/mL thrombin produced a time-dependent and significant increase in the polymerized tubulin content compared with that determined in the control period, while not many changes were detected in the total tubulin content (Fig. 5).

Furthermore, the effect of microtubule-disrupting agents on the thrombin-induced changes in $[Ca^{2+}]_i$ was investigated. When thrombin (1 unit/mL) was added to the medium containing fura 2-loaded endothelial cells, the fluorescence ratio (F_{340}/F_{360}) of the cells increased, indicating that an increase of $[Ca^{2+}]_i$ takes place with thrombin treatment. The elevation in fluorescence ratio in each cell was preceded by various time lags but reached the maximum level within a few seconds, in most cases. Pretreatment with either colchicine or vinblastine at a concentration of 10^{-6} M for 1 hr reduced the number of cells responding to thrombin.

DISCUSSION

IR-ET-1 secretion from porcine aortic endothelial cells was remarkably enhanced by stimulation with thrombin; the pattern of IR-ET-1 secretion was characterized by a slow onset and a gradual increase. Furthermore, it was demonstrated that thrombin-induced IR-ET-1 secretion was remarkably inhibited by treatment with either colchicine or vinblastine. It is known that both compounds have a high affinity for tubulin molecules and that both were effective in suppressing the polymerization of tubulin molecules. The polymerization of tubulin molecules is essential for the formation of microtubules. The inhibitory effect of colchicine and vinblastine on the secretion of various active substances from the secretory cells has been ascribed to their property of inhibiting the polymerization of tubulins [13–16]. Accordingly, it was assumed that the inhibitory effect of colchicine and vinblastine on the IR-ET-1 secretion may be caused by an inhibitory effect of these substances on microtubule formation, since

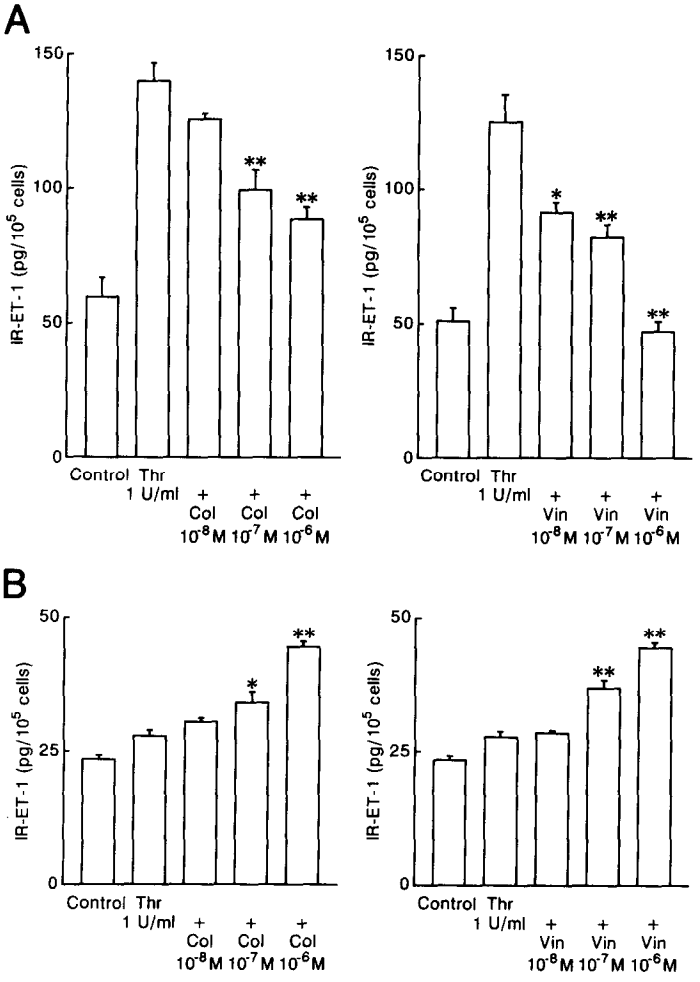

Fig. 2. Effect of colchicine (Col) and vinblastine (Vin) on IR-ET-1 secretion induced by thrombin (Thr) (A) and on the intracellular IR-ET-1 content of porcine aortic endothelial cells (B). The endothelial cells were incubated with Thr in the presence of Col or Vin for 6 hr. Each column and bar represent mean ± SEM of 4–6 experiments. *, **; Significantly different from the Thr-treated group at $P < 0.05$ and $P < 0.01$, respectively.

lumicolchicine, an analog of colchicine which does not have the ability to bind tubulin, did not affect IR-ET-1 secretion. It was also shown that these two agents are effective in inhibiting the basal secretion of ET-1. Furthermore, colchicine and vinblastine caused an increase in the intracellular content of IR-ET-1 in cells treated with thrombin, especially in the mitochondrial and microsomal fractions. Moreover, cycloheximide and actinomycin D are effective not only in inhibiting thrombin-induced IR-ET-1 secretion, but also in inhibiting the intracellular accumulation of IR-ET-1. This observation also supports the assumption that ET-1 release from the endothelial cells is largely dependent on the production of ET-1. It was assumed that the inhibition of tubulin polymerization, resulting in the impairment of microtubules, may be intimately related to the inhibition of ET-1 secretion rather than to the suppression of ET-1 synthesis. It was also assumed that the microtubules play some important roles in transporting the secretory granules and the Golgi-derived vesicles towards the plasma membrane in the secretory cells [17, 18]. In fact, in the present study, it was indicated that thrombin produced an increase in the polymerized tubulin content without affecting the total tubulin content, suggesting that the assembly of microtubules is a requisite for ET-1 secretion. These results suggest that the microtubular system may be involved in ET-1 secretion, especially in the process of transferring the synthesized ET-1 to the cell surface, even in basal secretion. Nakamura *et al.* [19] have demonstrated that immunoreactive endothelin is specifically localized in the rough endoplasmic reticulum (RER), Golgi cisternae, Golgi vesicles and small vesicles beneath the cell membrane of the cultured endothelial cells, suggesting that ET-1 synthesized in the RER is transported to Golgi complexes, packed into Golgi vesicles and secreted by exocytosis. Recently, it has been reported that the conversion of big ET-1, an intermediate precursor of ET-1, to mature ET-1, occurs in the intracellular

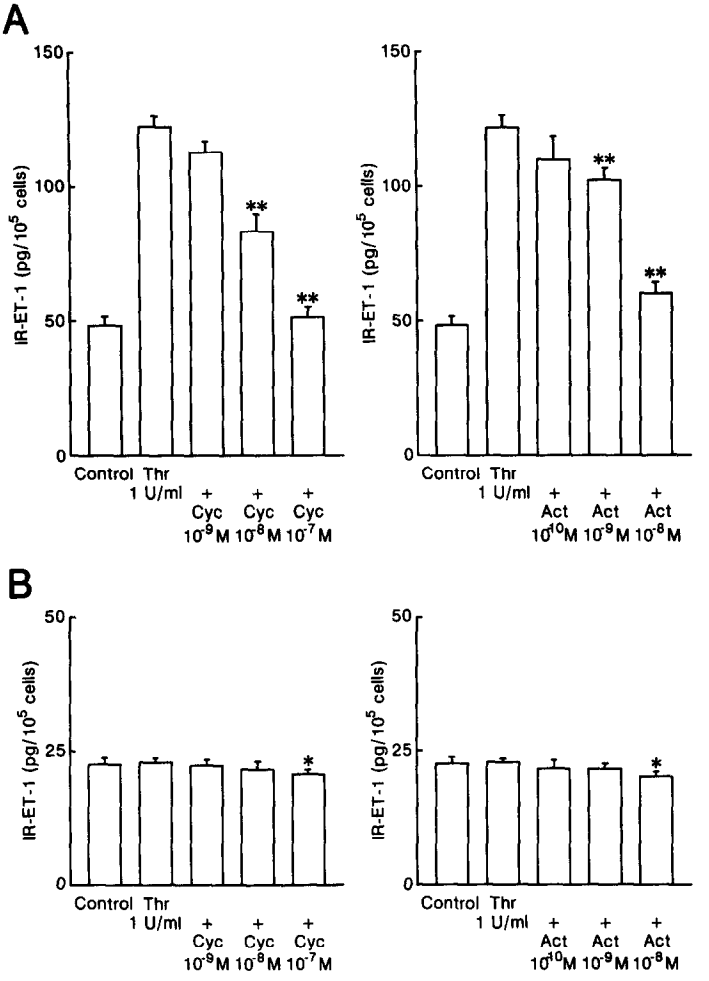

Fig. 3. Effect of cycloheximide (Cyc) and actinomycin D (Act) on IR-ET-1 secretion induced by thrombin (Thr) (A) and on the intracellular IR-ET-1 content of porcine aortic endothelial cells (B). The endothelial cells were incubated with Thr in the presence of Cyc or Act for 6 hr. Each column and bar represents mean ± SEM of 4–6 experiments. *, **; Significantly different from the Thr-treated group at $P < 0.05$ and $P < 0.01$, respectively.

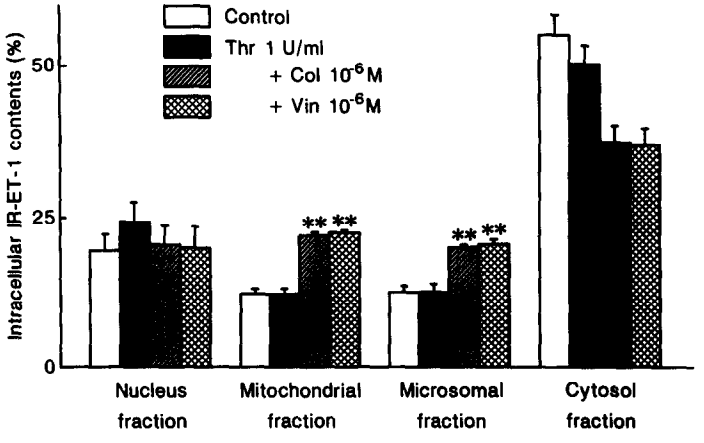

Fig. 4. Influence of colchicine (Col) and vinblastine (Vin) on intracellular distribution of IR-ET-1 in the thrombin (Thr)-treated porcine aortic endothelial cells. The endothelial cells were incubated with Thr in the presence or absence of Col or Vin for 6 hr. Each column and bar represents mean ± SEM of 4 experiments. **; Significantly different from the Thr-treated group at $P < 0.01$.

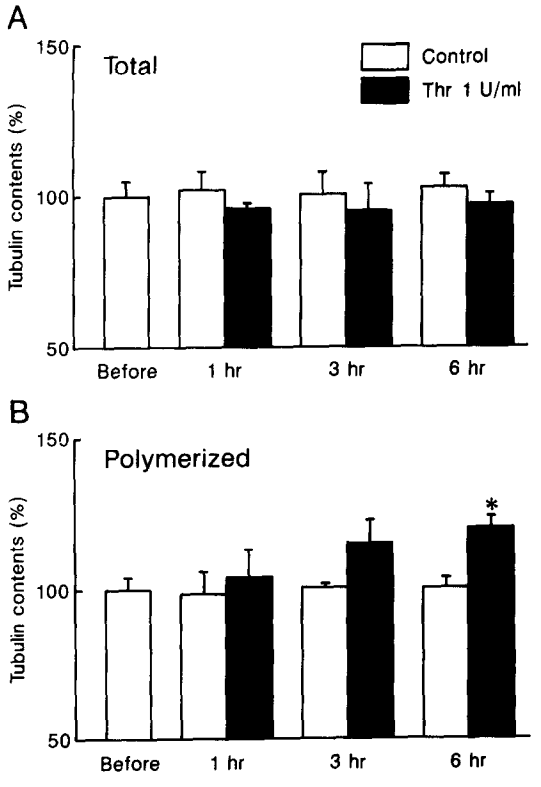

Fig. 5. Effect of thrombin (Thr) on the total (A) and polymerized tubulin (B) contents of porcine aortic endothelial cells. The endothelial cells were incubated with Thr 1 unit/mL. Each column and bar represents mean ± SEM of 5 experiments and these are expressed as percentage of tubulin content determined before Thr application. *; Significantly different from the control group at $P < 0.05$.

component of endothelial cells, since phosphoramidon, an endothelin converting enzyme inhibitor, inhibits both the intracellular conversion of big ET-1 to ET-1 in endothelial cells and the release of ET-1 from endothelial cells [20]. This indicates that ET-1 is released in the mature form from the endothelial cells. From these findings, it is suggested that ET-1 is synthesized and matured in the intracellular components of endothelial cells and secreted through the microtubular system. Furthermore, it was confirmed that even in the basal secretion, the microtubular system plays essential roles in the transportation of ET-1.

On the other hand, it was also demonstrated that thrombin caused an elevation in $[Ca^{2+}]_i$, and that the elevation was inhibited by colchicine or vinblastine. Ionomycin, a Ca^{2+} ionophore, has been shown to cause a marked and rapid induction of preproendothelin mRNA and the enhancement of ET-1 secretion from endothelial cells [7, 21]. These findings indicate that the elevation in $[Ca^{2+}]_i$ may be intimately related to the ET-1 secretion induced by thrombin. It has previously been shown that microtubules play an important role in histamine release from rat peritoneal mast cells, especially in Ca^{2+} release from the intracellular Ca store [22]. From these findings, it is suggested that microtubules may participate in ET-1 secretion, not only in the intracellular transport of ET-1 but also in the process leading to Ca^{2+} release from the intracellular Ca store in endothelial cells which, in turn, is an essential trigger for the release of active substances in many secretory cells.

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