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THE ROLE OF c-JUN PROTEIN IN THROMBIN-STIMULATED EXPRESSION OF PREPROENDOTHELIN-1 mRNA IN PORCINE AORTIC ENDOTHELIAL CELLS

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Abstract—Treatment of porcine aortic endothelial cells with thrombin induced a time- and dose-dependent expression of preproendothelin-1 (PPET-1) mRNA. The thrombin-induced expression of PPET-1 mRNA was markedly inhibited by calphostin C, a specific inhibitor of protein kinase C, and phorbol 12-myristate 13-acetate (TPA) induced the expression of PPET-1 mRNA dose-dependently, but 4α-phorbol 12, 13-didecanoate, an inactive enantiomer of phorbol ester, had no effect on the expression of PPET-1 m RNA. On the other hand, challenge of the endothelial cells with thrombin induced a marked and time-dependent increase in the binding activity of nuclear extract to the TPA-responsive element. Furthermore, thrombin elicits synthesis of c-Jun protein as well as triggering its dephosphorylation. From these results, it is concluded that thrombin-stimulated expression of PPET-1 mRNA in porcine aortic endothelial cells can be induced not only by c-Jun protein synthesis but also by initial dephosphorylation in response to activation of protein kinase C.

Endothelin-1 (ET-1†) is a potent vasoconstricting peptide consisting of 21-amino-acid residues originally identified in culture supernatants of porcine aortic endothelial cells [1]. Recently, it became clear that ET-1 possesses a wide variety of biological activities in addition to its vasoconstrictor action [2]. ET-1 is derived from a precursor consisting of approximately 200 residues, preproendothelin-1 (PPET-1), via an intermediate consisting of 38 (human) or 39 (porcine) residues known as big ET-1 [1, 3]. Conversion from big ET-1 to ET-1 takes place through processing by endothelin-converting enzyme at Trp^{21}-Val^{22} [1, 3]. It has been shown that ET-1 is secreted by endothelial cells in response to various chemical and mechanical stimuli such as thrombin, transforming growth factor-β (TGF-β), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), Ca^{2+} ionophore and hemodynamical shear stress [4–10]. We have previously shown that the microtubular systems are important in ET-1 secretion by endothelial cells [5]. Furthermore, it has recently been demonstrated that phosphorylation of myosin light chain, elicited by myosin light chain kinase, facilitates the formation of filamentous myosin and actin which are probably involved in ET-1 secretion, possibly in the transport of ET-1-containing vesicles in thrombin-stimulated endothelial cells [11]. Since only a few secretory granules could be seen in the endothelial cells, and since the level of PPET-1 mRNA in the cells increased after exposure to the stimulus eliciting ET-1 secretion, the secretion of ET-1 may be regulated at the level of mRNA transcription [1, 6–8, 10]. The present study was undertaken to investigate the regulation of thrombin-induced expression of PPET-1 mRNA in porcine aortic endothelial cells.

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† Abbreviations: ET-1, endothelin-1; IR-ET-1, immunoreactive ET-1; PPET-1, preproendothelin-1; TGF-β, transforming growth factor β; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; RIPA, radioimmunoprecipitation assay; EGTA, ethyleneglycol-bis(β-aminoethyl ether)*N,N,N',N'*,-tetraacetic acid; TPA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; TRE, TPA-responsive element; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; W-7, *N*-(6-amino-hexyl)-5-chloro-1-naphthalenesulfonamide; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine; CAT, chloramphenicol acetyltransferase.

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, Japan) supplemented with 10% fetal calf serum, 100 U/mL penicillin (Meiji, Japan) and 100 μg/mL streptomycin (Meiji, Japan) in a humidifed atmosphere of 5% CO_2/95% air as described previously [5]. The cells were subcultured in 60-mm dishes for 3–4 days and allowed to grow into confluent monolayers. Confluent cultures of endothelial cells were fed with serum-free DMEM containing 0.1% fatty acid-free bovine serum albumin for 6 hr prior to the experiment. The ET-1 content of the culture medium was determined by radioimmunoassay using a specific antiserum for ET-1 (Peptide Institute, Osaka, Japan) and [^{125}I]-ET-1 (Amersham, U.K.) as a tracer as described previously [5].

Northern blot analysis. After the test compound

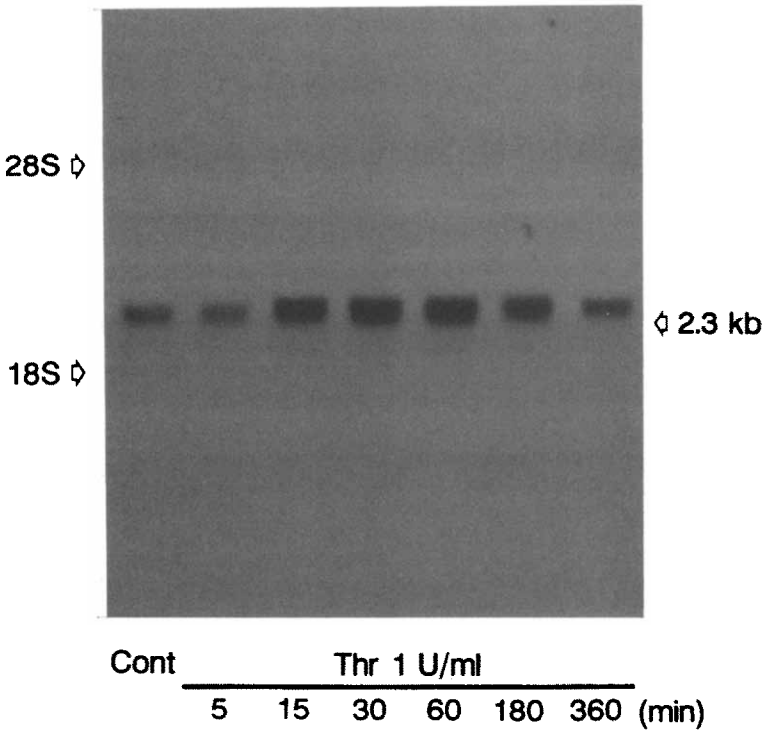

Fig. 1. Sequential changes in expression of PPET-1 mRNA induced by thrombin in porcine aortic endothelial cells. The endothelial cells were cultured to confluence in 60-mm dishes and incubated with thrombin at a concentration of 1 U/mL for various periods of time. The total cellular RNA (5 μg/lane) was electrophoresed, blotted and hybridized with the [^{32}P]5′ end-labeled 63-mer oligodeoxynucleotide probe complementary to the mRNA coding for Cys^{1}-Trp^{21} of ET-1. Ribosomal RNAs (18S and 28S) were used as size markers. Cont, control; Thr, thrombin.

had been present in the medium for various periods of time, total cellular RNA was extracted by a mini-prep method [12]. Total cellular RNA (5 μg/lane) was denaturated by heating in 1 M glyoxal, 50% dimethyl sulfoxide and 10 mM sodium phosphate (pH 7.0) at 50° for 1 hr. Denaturated RNA was placed on 1.2% agarose gels, electrophoresis was performed and then the RNA was transferred to Hybond-N+ nylon membranes (Amersham, U.K.) by capillary elution using 50 mM NaOH as a transfer buffer. All samples were applied to gels in duplicate and another gel was stained with ethidium bromide to visualize 18S and 28S ribosomal RNA bands. These bands were used to confirm that equivalent amounts of RNA were loaded in each gel lane and that no obvious degradation of RNA took place. The blotted membrane was incubated for 4 hr at 42° in prehybridization buffer consisting of 5 × SSPE (20 × stock solution: 3.6 M NaCl, 200 mM sodium phosphate (pH 7.7), 20 mM Na_2EDTA), 5 × Denhardt's solution, 50% formamide, 0.5% sodium dodecyl sulfate (SDS) and 100 μg/mL salmon sperm DNA. For detecting PPET-1 mRNA, synthetic oligodeoxyribonucleotide 5′-CCA GAT GAT GTC CAG GTG GCA GAA GTA GAC ACA CTC TTT ATC CAT CAG GGA AGA GCA GGA GCA-3′ (Kurabo, Japan), which is complementary to the coding sequence for Cys^{1}-Trp^{21} of porcine ET-1, was used as the probe. Hybridization was conducted for 24 hr at 42° in prehybridization buffer containing the ^{32}P-labeled oligonucleotide probe which was phosphorylated by T4 polynucleotide kinase (Mega-label, Takara, Japan). After hybridization, the membrane was washed three times with 2 × SSPE and 0.1% SDS for 15 min at 42°, once with 1 × SSPE and 0.1% SDS for 30 min at 42°, and twice with 0.1 × SSPE and 0.1% SDS for 15 min at room temperature. The membrane was placed on filter paper, sealed in a plastic bag and exposed to Hyperfilm-MP (Amersham) at −80°, or exposed to an imaging plate (Fuji) and analysed by a bio-image analyser (BAS 2000, Fuji).

Gel mobility shift assay. A 20-base double-stranded synthetic DNA fragment was prepared that contained the TPA-responsive element of the PPET-1 gene [13]. The sequence was as follows:

5′-CTGTTGGTGACTAATAACAC-3′
3′-GACAACCACTGATTATTGTG-5′.

After the compound had been added to the medium for various periods of time, nuclear extracts from endothelial cells were prepared by the method of Schreiber *et al.* [14]. The nuclear extract (5 μg of protein) was added to 20 μL of reaction mixture containing 20 mM HEPES (pH 7.9), 80 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.3 mM

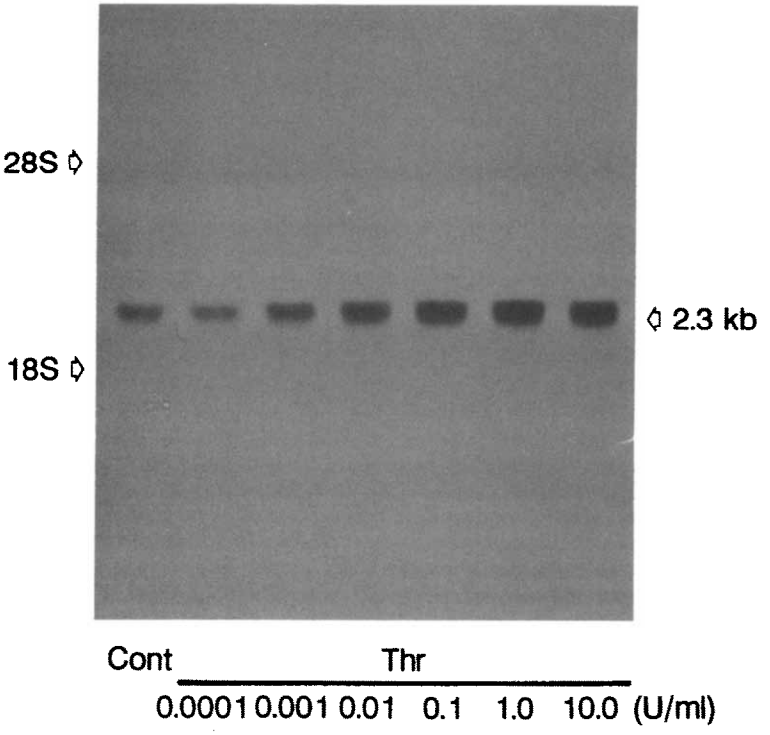

Fig. 2. Dose-related expression of PPET-1 mRNA induced by thrombin in porcine aortic endothelial cells. The endothelial cells were cultured to confluence in 60-mm dishes and incubated with thrombin for 30 min at various concentrations. The total cellular RNA (5 μg/lane) was electrophoresed, blotted and hybridized with the [^{32}P]5′ end-labeled 63-mer oligodeoxynucleotide probe complementary to the mRNA coding for Cys^{1}-Trp^{21} of ET-1. Ribosomal RNAs (18S and 28S) were used as size markers. Cont, control; Thr, thrombin.

phenylmethylsulfonyl fluoride, 10% glycerol, 2 μg poly(dI-dC) (Pharmacia) and kept at 0° for 5 min. To this mixture, a ^{32}P-labeled DNA fragment (approximately 0.4 ng 10,000 cpm) phosphorylated by T4 polynucleotide kinase was added. After a 30 min incubation at 27°, 1 μL of loading buffer (0.05% Bromophenol blue, 5% glycerol and 50 mM EDTA) was added. Samples were applied to a 4% polyacrylamide gel (acrylamide/bisacrylamide, 29:1) which was dissolved in TAE buffer (6.7 mM Tris–HCl (pH 7.5), 3.3 mM sodium acetate and 1 mM EDTA). The gel was prerun at 11 V/cm at 4° for 1 hr recirculating the TAE buffer, and run at 11 V/cm at 4° for 2.5 hr continuously recirculating with the same buffer. The gel was dried and exposed to Hyperfilm-MP at −80°, or exposed to an imaging plate and analysed by a bio-image analyser.

Immunoprecipitation. After the endothelial cells had grown to confluence in 60-mm dishes, the medium was changed to L-methionine-free DMEM. EXPRE[^{35}S][^{35}S] (0.2 mCi/mL, Dupont, Boston, MA) was added to the medium and incubation was continued at 37° for 5 hr. Thereafter, thrombin was added and the incubation was continued for various periods of time (5–60 min). After removal of the medium, the cell layer was washed once with ice-cold Tris-buffered saline (25 mM Tris–HCl (pH 7.4), 130 mM NaCl and 5 mM KCl), transferred to microcentrifuge tubes and centrifuged at 2000 *g* at 4° for 30 sec. The pellet was lysed in 500 μL of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl (pH 7.5), 400 mM NaCl, 4 mM EDTA, 4 mM EGTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.001% leupeptin and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 0°. The cell lysate was clarified by centrifugation at 16,000 *g* for 10 min at 4°. The supernatant was incubated with 25 μL of Protein G PLUS/Protein A-Agarose (Oncogene Science) for 1 hr at 0° and centrifuged at 16,000 *g* for 5 min at 4°, and the supernatant was incubated with 15 μL of affinity-purified IgG against c-Jun peptide (Oncogene Science) for 1 hr at 0°. The sample was then incubated with 25 μL of Protein G PLUS/Protein A-Agarose for 1 hr. The immunoprecipitate was collected by centrifugation at 16,000 *g* for 5 min at 4° and the pellet was washed three times with RIPA buffer and once with 10 mM Tris–HCl (pH 7.5). The pellet was then treated with 25 μL of sample buffer (40 mM Tris–HCl (pH 6.8), 40% glycerol, 2% β-mercaptoethanol and 2% SDS) and boiled for 5 min. Thereafter, the specimen was centrifuged at 16,000 *g* for 5 min at 4° and subjected to SDS–PAGE. The gel was dried and exposed to Hyperfilm-MP at −80°, or exposed to an imaging plate and analysed by a bio-imaging analyser.

Phosphorylation. Endothelial cells grown to confluence in 60-mm dishes were labeled by

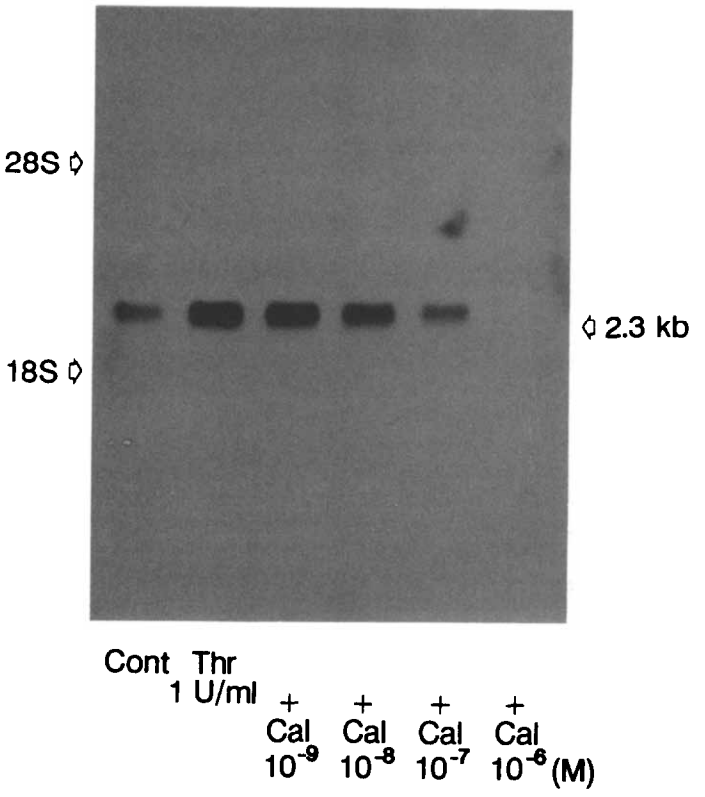

Fig. 3. Effect of calphostin C on the expression of PPET-1 mRNA induced by thrombin. Endothelial cells were cultured to confluence in 60-mm dishes and incubated with thrombin in the presence of calphostin C for 30 min. The total cellular RNA (5 μg/lane) was electrophoresed, blotted and hybridized with the [^{32}P]5′ end-labeled 63-mer oligodeoxynucleotide probe complementary to the mRNA coding for Cys^{1}-Trp^{21} of ET-1. Ribosomal RNAs (18S and 28S) were used as size markers. Cont, control; Thr, thrombin; Cal, calphostin C.

incubating with 0.2 mCi/mL of [^{32}P]orthophosphoric acid (Amersham) at 37° for 3 hr in 2 mL of phosphate-free DMEM. The cells were then washed three times with phosphate-free DMEM and preincubated for 30 min at 37° in the same buffer. Thereafter, thrombin was added and incubation was continued for various periods of time (5–60 min). Thereafter, the cells were lysed in 500 μL of RIPA buffer containing 10 mM sodium pyrophosphate, 10 mM NaF and 2 mM Na_3VO_4. Immunoprecipitation and SDS–PAGE were carried out as described before.

Chemicals. Thrombin (from bovine plasma), phorbol 12-myristate 13-acetate (TPA) and 4α-phorbol 12, 13-didecanoate (4α-PDD) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Calphostin C was kindly provided by the Kyowa Hakko Kogyo Co. (Japan). Other chemicals used were all reagent grade and were purchased from a commercial source.

RESULTS

To investigate the regulation of ET-1 gene expression in endothelial cells, the time course and dose-dependence of the induction of PPET-1 mRNA in cultured porcine aortic endothelial cells were studied after exposure to thrombin (Figs 1 and 2). When the endothelial cells were incubated in the presence of thrombin (1 U/mL), the PPET-1 mRNA (2.3 Kb) level had rapidly increased several-fold after 15 min. The expression of PPET-1 mRNA reached a peak about 30 min after the addition of thrombin, then began to decrease and was restored to the basal level after 6 hr. Thrombin enhanced the expression of PPET-1 mRNA in a dose-dependent manner at concentrations ranging from 0.01 to 10 U/mL. When calphostin C, a specific inhibitor of protein kinase C [15], was added to the medium at concentrations ranging from 10^{-9} to 10^{-6} M, simultaneously with thrombin (1 U/mL), the amount of PPET-1 mRNA decreased markedly and dose-dependently (Fig. 3). Treatment with TPA at concentrations ranging from 10^{-10} to 10^{-8} M elicited a dose-dependent increase in PPET-1 mRNA level (Fig. 4A). An inactive enantiomer of phorbol ester, 4α-PDD, had no effect on the expression of PPET-1 mRNA even at a concentration of 10^{-7} M (Fig. 4B), indicating that the effect of TPA was not exhibited by all phorbol esters. Furthermore, to confirm whether or not the induction of PPET-1 mRNA by thrombin is accompanied by the secretion of immunoreactive ET-1 (IR-ET-1) into the medium, changes in the rate of IR-ET-1 secretion were determined in the presence and in the absence of

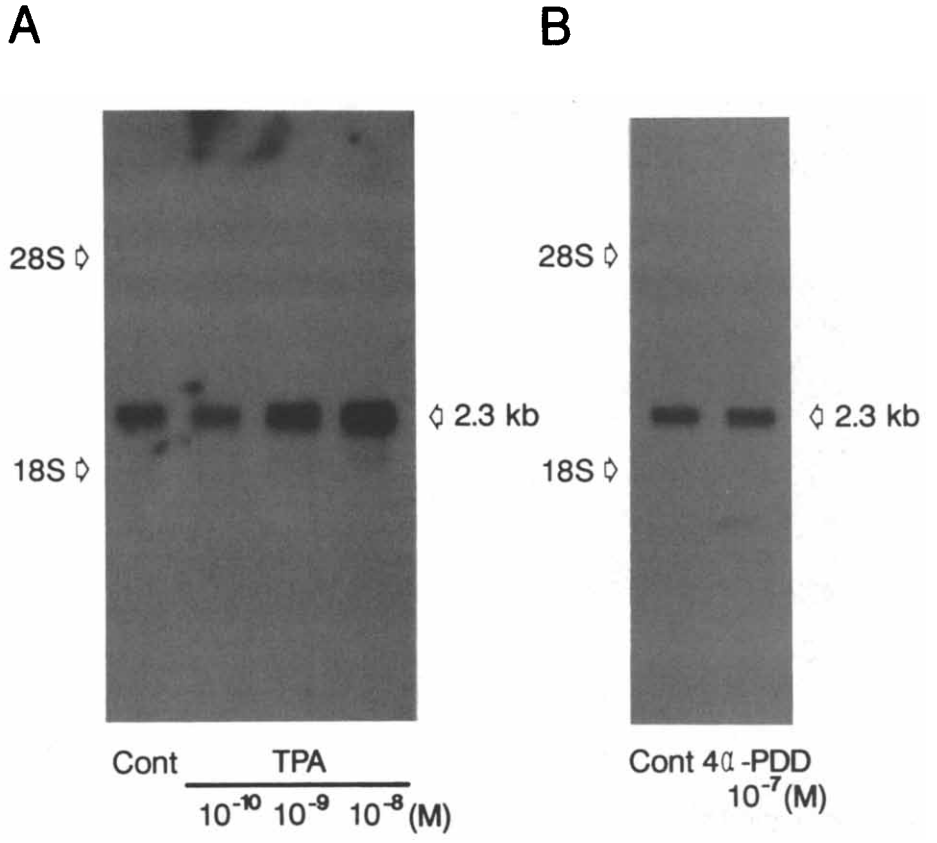

Fig. 4. Effects of phorbol esters on PPET-1 mRNA levels in endothelial cells (A, B). The endothelial cells were cultured to confluence in 60-mm dishes and incubated with phorbol esters for 30 min (A, B). The total cellular RNA (5 μg/lane) was electrophoresed, blotted and hybridized with the [^{32}P]5′ end-labeled 63-mer oligodeoxynucleotide probe complementary to the mRNA coding for Cys^{1}-Typ^{21} of ET-1. Ribosomal RNAs (18S and 28S) were used as size markers. Cont, control.

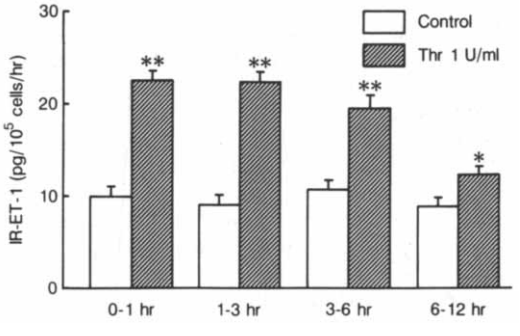

Fig. 5. Changes in the rate of IR-ET-1 secretion by endothelial cells after treatment with thrombin (Thr). The endothelial cells were cultured to confluence in a 24-well plate and incubated in the presence and absence of thrombin at a concentration of 1 U/mL. Each column and bar represent the mean ± SEM of five experiments. *, **, Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

thrombin (Fig. 5). In the control group, there was no change in the rate of IR-ET-1 secretion and this basal level persisted for 12 hr. When the endothelial cells were treated with thrombin at a concentration of 1 U/mL, the rate of IR-ET-1 secretion increased to twice that of the control within the 1st hr and this level was maintained until the 6th hr. From the 6th to the 12th hr, the secretion rate decreased and remained at about 1.3-fold that of the untreated cells.

The 5′-flanking region of the PPET-1 gene contains an octanucleotide sequence for the TPA-responsive element (TRE) and this region is also known as the binding element for c-Jun and the c-Jun–c-Fos complex [13]. To determine whether thrombin treatment is able to increase the binding activity of nuclear extracts to the TRE, a gel mobility shift assay was performed. As shown in the control of Fig. 6, a single retarded band, representing a TRE-protein complex, was observed even when the nuclear extract of non-treated cells was employed as the protein portion. When the endothelial cells were treated with thrombin at a concentration of 1 U/mL the amounts of TRE–protein complexes increased markedly and time dependently. The addition of an

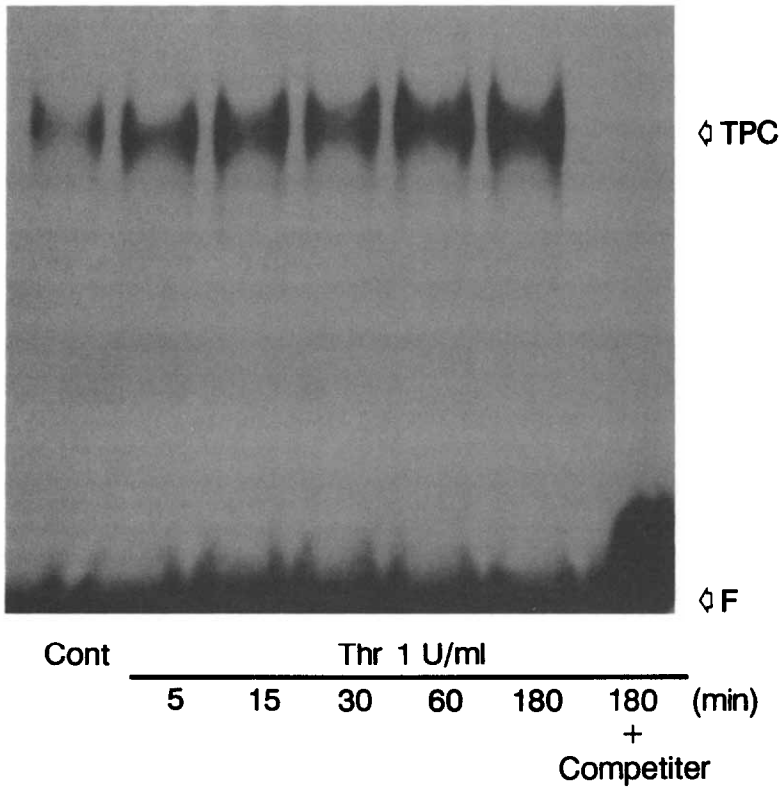

Fig. 6. Induction of TRE binding activity in the nuclear extract of endothelial cells treated with thrombin. The endothelial cells were cultured to confluence in 60-mm dishes and were incubated with thrombin (1 U/mL) for various periods of time. Nuclear extract obtained from endothelial cells was incubated with ^{32}P-labeled TRE consensus fragment. Excess amount (approximately 100-fold) of unlabeled DNA fragment was used for competition. The gel mobility shift assay was performed in 4% polyacrylamide gel. TPC, TRE–protein complex; F, free DNA fragment; Cont, control; Thr, thrombin.

excess amount (about 100-fold) of unlabeled DNA fragment containing the TRE prevented formation of the radioactive binding complex. These results indicate that the gel mobility shift is due to specific TRE–protein interaction. Furthermore, when affinity-purified IgG against c-Jun peptide was added to the reaction mixture of the synthetic DNA fragment and nuclear extract, we observed another more retarded band in the gel mobility shift assay (Fig. 7). However, no such reaction was observed when normal rabbit serum was used in this assay.

Since the anti-c-Jun peptide antibody induced an immunoreaction with the TRE–protein complex, we examined further the ability of ^{35}S-labeled endothelial cells to produce c-Jun protein by means of immunoprecipitation using the anti-c-Jun peptide antibody (Fig. 8A and B). In the control cells, a visible but thin protein band was observed with an apparent molecular mass of 39 kDa. However, when the cells were treated with thrombin (1 U/mL), the 39 kDa band became more evident and three other protein bands of 42, 46 and 55 kDa also became evident. Based on the molecular mass and its capability to form a heterodimer with c-Jun protein in the presence of anti-c-Jun peptide antibody, it was assumed that the 55 kDa protein might be c-Fos protein [16–18]. The amount of 39 kDa protein gradually increased and reached a maximum at 30 min after thrombin treatment. The other protein bands were observed at 5 min and then gradually decreased during the subsequent 60 min. The thrombin-induced increases in these protein bands were inhibited by calphostin C at a concentration of 10^{-6} M. None of these proteins was immunoprecipitated when normal rabbit serum was used (data not shown). However, it is known that the DNA binding activity of c-Jun protein is regulated by phosphorylation [19–22]. Thus, we examined by means of the RIPA whether or not the phosphorylation of c-Jun protein takes place in thrombin-treated endothelial cells. The results shown in Fig. 9A and B indicate that the 39-kDa phosphorylated protein was included in the immunoprecipitate combined with anti-c-Jun peptide antibody and the control cell lysate. When the endothelial cells were treated with thrombin (1 U/mL), rapid dephosphorylation of the 39-kDa protein took place within 5 min. This initial dephosphorylation was restored to the control level at 15 min after the addition of thrombin. Pretreatment of the cells with calphostin C at a concentration of 10^{-6} M inhibited the thrombin-induced dephosphorylation. No phosphorylated proteins were immunoprecipitated with normal rabbit serum (data not shown).

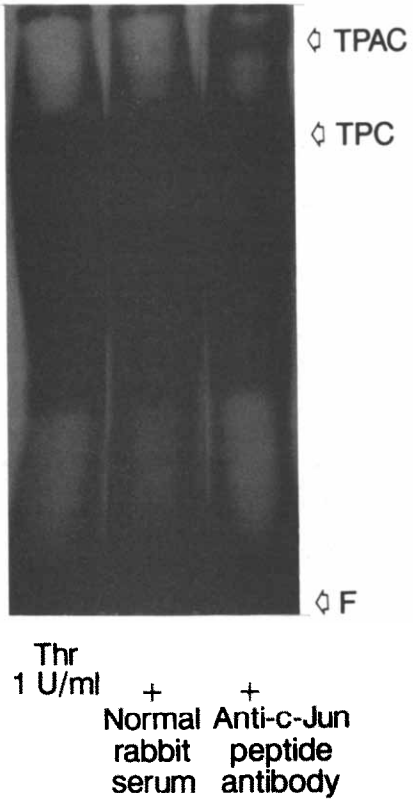

Fig. 7. Immunological identification of c-Jun in the TRE–protein complex. Endothelial cells were cultured to confluence in 60-mm dishes. Anti-c-Jun peptide antibody or normal rabbit serum was added to the mixture of ^{32}P-labeled TRE consensus fragment and nuclear extracts obtained from endothelial cells treated with thrombin (1 U/mL) for 30 min. The gel mobility shift assay was performed in 4% polyacrylamide gel. TPC, TRE–protein complex; F, free DNA fragment; TPAC, TRE–protein–antibody complex; Thr, thrombin.

DISCUSSION

It has been shown that thrombin stimulates the secretion of ET-1 by porcine aortic endothelial cells, and that the onset of the thrombin-induced increase in ET-1 secretion is earlier than that of TGF-β, IL-1 and TNF-α [4–8]. It has been reported that treatment of porcine aortic endothelial cells with thrombin for 1 hr induces an increase in PPET-1 mRNA [11]. However, changes in the level of PPET-1 mRNA during the 1st hr have not been examined. In the present study, the expression of PPET-1 mRNA in porcine aortic endothelial cells was markedly enhanced by stimulation with thrombin at 15 min, and the time course of expression of PPET-1 mRNA was characterized by faster onset than in the case of TGF-β, IL-1 and TNF-α [6–8]. Furthermore, it was confirmed that the expression of PPET-1 mRNA induced by thrombin was followed by ET-1 secretion by endothelial cells as shown in Fig. 5. The thrombin-induced expression of PPET-1 mRNA was inhibited by calphostin C, an inhibitor of protein kinase C, at concentrations ranging from 10^{-8} to 10^{-6} M [15]. Calphostin C is a potent and specific protein kinase C inhibitor, acting on the regulatory domain of protein kinase C [15]. The IC_{50} of calphostin C for inhibiting protein kinase activity was reported to be 5×10^{-8} M [15]; this value seems to correspond to that seen for thrombin-induced expression of PPET-1 mRNA (Fig. 3). Furthermore, TPA, an activator of protein kinase C, enhanced the expression of PPET-1 mRNA, while 4α-PDD, an inactive enantiomer of phorbol ester, had no influence on the expression of PPET-1 mRNA. Inoue *et al.* [13] also tested the effects of phorbol esters on the expression of PPET-1 mRNA in cultured human endothelial cells. On addition of 0.5 μM TPA to the medium, PPET-1 mRNA was rapidly induced several-fold within 10 min, while the inactive enantiomer of phorbol ester, 4α-PDD, had no effect. Although 1 nM of TPA was effective in inducing the expression of PPET-1 mRNA in the present experiment, 4α-PDD had no effect. These results indicate that the thrombin-induced expression of PPET-1 mRNA is regulated by intracellular signaling mediated by protein kinase C.

In a previous study, it was shown that when the endothelial cells were treated with thrombin, the phosphorylation of myosin light chain occurred [11]. Drugs, such as TMB-8, W-7 and ML-9, which inhibit the phosphorylation, are all capable of preventing IR-ET-1 secretion [11]. At a concentration of 10^{-5} M, calphostin C did not inhibit either the thrombin-induced phosphorylation or thrombin-induced IR-ET-1 secretion [11]. As shown in Fig. 3, the expression of PPET-1 mRNA in endothelial cells was completely inhibited by calphostin C at 10^{-6} M in the present experiment. These findings indicate that the secretion of ET-1 by endothelial cells may be regulated not only at the level of transcription, but also at various steps following the transcriptional stage. Actually, it was demonstrated that the microtubular systems and the formation of polymerized actin and myosin through phosphorylation of myosin light chain seem to be intimately involved in ET-1 secretion [5, 11]. As previously shown [5], when the microtubular systems were damaged, IR-ET-1 secretion was inhibited but the intracellular content of IR-ET-1 was significantly increased. This suggests that if the microtubular systems are not destroyed, the secretion of presynthesized ET-1 is induced by thrombin treatment even after the content of PPET-1 mRNA has been decreased by calphostin C.

It has been shown that both the TRE binding activity *in vitro* and transcriptional properties *in vivo* are enhanced by treatment with TPA, which is known to exert its biological effects via activation of protein kinase C [23, 24]. The PPET-1 gene contains the TREs, which are recognized by c-Jun homodimer and c-Jun–c-Fos heterodimer, in the 5′-flanking region [13]. Treatment of endothelial cells with thrombin induced an increase in TRE binding activity in nuclear extracts, suggesting that the activity of the TRE recognition factor is increased. Furthermore, it has been shown that this TRE–protein complex contains c-Jun protein as seen in the gel mobility shift assay using anti-c-Jun peptide antibody. The c-Jun protein is a major component

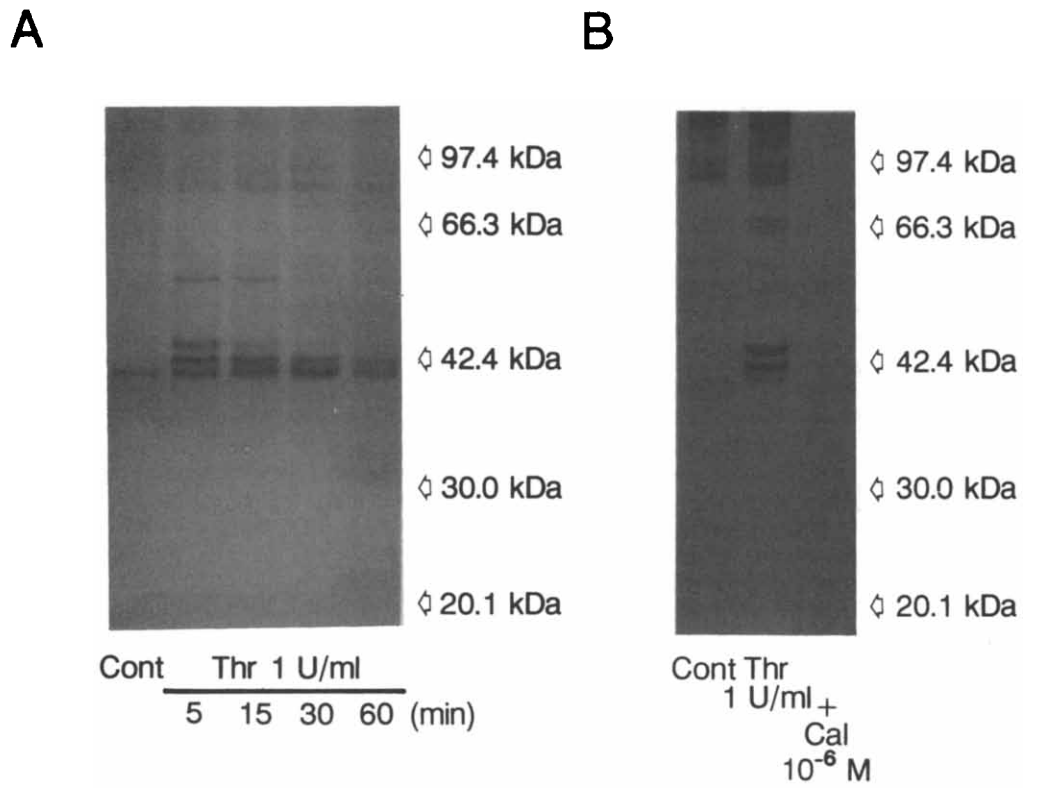

Fig. 8. Time course of c-Jun protein synthesis induced by thrombin (A) and the effect of calphostin C on the thrombin-induced synthesis of c-Jun protein in endothelial cells (B). The ^{35}S-labeled endothelial cells were incubated with thrombin (1 U/mL) for 5, 15, 30 or 60 min (A), and 5 min after preincubation with calphostin C for 30 min (B). The level of c-Jun protein synthesis was estimated by radioimmunoprecipitation of lysates obtained from an equal number of cells using anti-c-Jun peptide antibody. Cont, control; Thr, thrombin; Cal, calphostin C.

of the transcription factor AP-1 [25, 26], which mediates TPA-induced expression of responsive genes through the TRE [24, 27]. Actually, the increase in TRE binding activity was followed by the expression of PPET-1 mRNA, though there was a time lag between these two phenomena. These results suggest that thrombin-induced expression of PPET-1 mRNA may be mediated by the binding of c-Jun protein to the TRE.

Immunoprecipitation of thrombin-stimulated ^{35}S-labeled endothelial cell with anti-c-Jun peptide antibody revealed the existence of a number of associated proteins ranging in molecular size from 39 to 55 kDa. On the basis of molecular mass, the 39- and 42-kDa proteins were identified as c-Jun protein and modified c-Jun protein, respectively [16, 28, 29]. The increase in c-Jun proteins was induced rapidly by thrombin prior to the induction of PPET-1 mRNA. The c-Jun protein forms homo- and heterodimers with Fos families which bind the TRE, but Fos proteins are only active as heterodimers with Jun proteins [30, 31]. Therefore, the 46- and 55-kDa proteins, which were co-precipitated with anti-c-Jun peptide antibody in the present study, seem to be of the same molecular size as Fra-2 protein and c-Fos protein, respectively [16–18, 31]. In the endothelial cells stimulated with thrombin, induction of c-Fos protein (55 kDa) and Fra-2 protein (46 kDa) was clearly enhanced in the same way as c-Jun protein (39 kDa). The pattern of induction of these proteins is similar to that in serum-stimulated NIH 3T3 cells [16]. In the presence of c-Fos, c-Jun prefers to associate with c-Fos, rather than with c-Jun itself, indicating that higher affinity stimulates the formation of c-Jun–c-Fos heterodimers rather than c-Jun homodimers [30]. Furthermore, it has been reported that the heterodimer binds to the TRE more efficiently than does the c-Jun homodimer [30]. Lee *et al.* [32] identified the AP-1 binding element in the promoter region of the PPET-1 gene and showed that site-directed mutagenesis of this AP-1 site results in a substantial reduction in promoter activity in the bovine aortic endothelial cells transfected with a chloramphenicol acetyltransferase (CAT) reporter construct. These workers have also reported that cotransfection with the AP-1/CAT reporter construct and c-*jun* and c-*fos* expression plasmids markedly increases the transcriptional rate of CAT reporter plasmids containing three synthetic ET-1 AP-1 sites [32]. Furthermore, as shown in Fig. 8, the thrombin-induced increase in c-Jun protein and c-Fos protein was inhibited by calphostin C at a concentration of 10^{-6} M, which is also effective in inhibiting the induction of PPET-1 mRNA. These

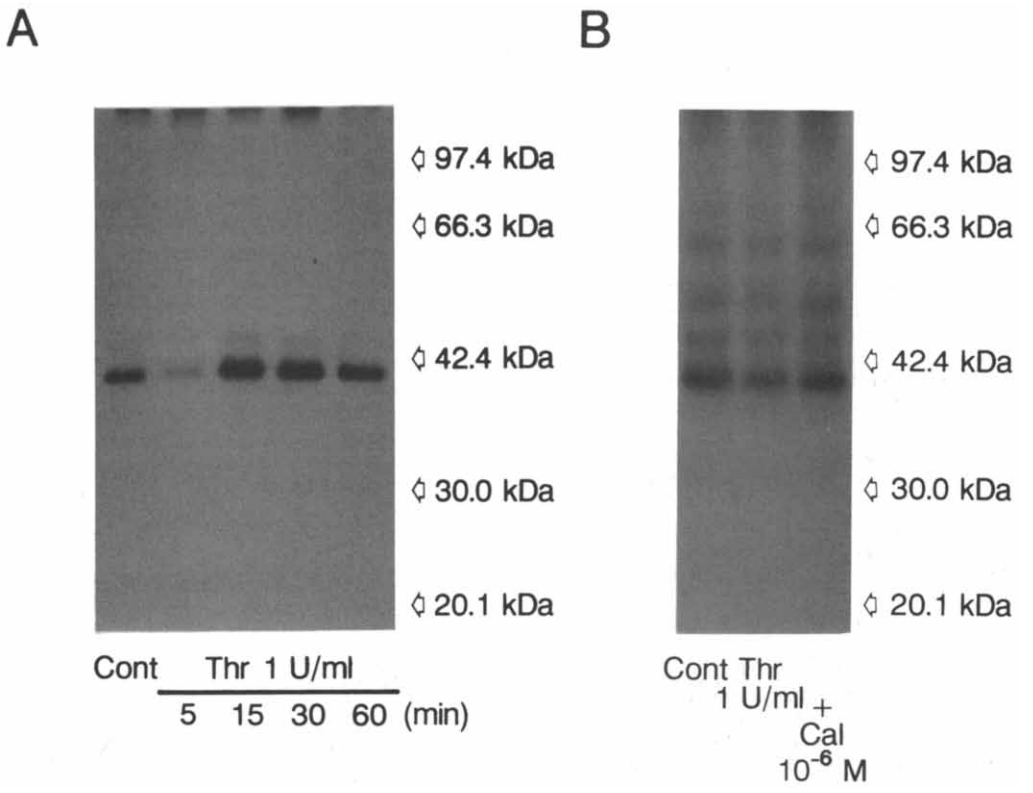

Fig. 9. Time course of dephosphorylation of c-Jun protein induced by thrombin (A) and the effect of calphostin C on the thrombin-induced dephosphorylation of c-Jun protein in endothelial cells (B). The ^{32}P-labeled endothelial cells were incubated with thrombin (1 U/mL) for 5, 15, 30 or 60 min (A), and 5 min after preincubation with calphostin C for 30 min (B). The level of phosphorylation was estimated by radioimmunoprecipitation of lysates obtained from an equal number of cells using anti-c-Jun peptide antibody. Cont, control; Thr, thrombin; Cal, calphostin C.

findings suggest that thrombin may induce expression of PPET-1 mRNA via an increase in c-Jun protein, followed by formation of the c-Jun–c-Fos heterodimer. In 1321N1 astrocytoma cells, thrombin was shown to induce a biphasic increase in c-*jun* mRNA, with an initial peak at 0.5 hr and a second, more prolonged increase at 12 hr [33]. It was also demonstrated that a secondary sustained increase in c-*jun* mRNA was necessary to cause a marked induction of c-Jun protein, AP-1 DNA binding activity and AP-1-mediated transactivation in 1321N1 cells, while the early transient induction of c-*jun* mRNA was not effective [33]. However, in porcine aortic endothelial cells, thrombin caused an increase in c-Jun protein and TRE binding activity much faster than in astrocytoma cells, indicating that in endothelial cells thrombin may be effective in producing a rapid but sufficient increase in c-*jun* mRNA. Immunoprecipitation of ^{32}P-labeled cultures indicates that the 39-kDa c-Jun protein is a phosphorylated protein (Fig. 9A). c-Jun protein is rapidly dephosphorylated in response to thrombin. The thrombin-induced dephosphorylation of c-Jun protein is inhibited by calphostin C at a concentration of 10^{-6} M, which is also effective in inhibiting the induction of PPET-1 mRNA. In resting cells, c-Jun protein is present in an inactive form which is phosphorylated by glycogen synthase kinase-3 at three residues adjacent to the DNA binding domain [19]. It is also known that activation of protein kinase C results in rapid dephosphorylation of c-Jun protein at these sites and, as a consequence, the binding activity of c-Jun protein to TRE increases [19]. It has also been reported recently that TPA treatment of human osteosarcoma (MG63) and epithelial (HeLa) cells leads to a net decrease in c-Jun phosphorylation 30 min later [19]. However, in the present experiment, dephosphorylation of c-Jun was clearly observed 5 min after exposure to thrombin. This may promote further expression of PPET-1 mRNA.

From these results, it is suggested that thrombin-stimulated expression of PPET-1 mRNA is induced by c-Jun protein synthesis and dephosphorylation in response to protein kinase C activation.

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