Endothelin-1 Enhances Plasminogen Activator Inhibitor-1 Production by Human Brain Endothelial Cells via Protein Kinase C-Dependent Pathway

Raphael Zidovetzki, Jin-Lin Wang, Jeong A. Kim, Peijia Chen, Mark Fisher, Florence M. Hofman

Abstract—The effects of endothelin-1 (ET-1) on the production of plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA) by human brain-derived endothelial cells in culture were studied. At 100 nmol/L, ET-1 increased PAI-1 production by 88±6% within 72 hours, and increased PAI-1 mRNA expression within 1 hour of stimulation; there was no significant effect on t-PA production. PAI-1 activity was also examined and found to increase with ET-1 treatment. Suboptimal concentrations of ET-1 and tumor necrosis factor-α (TNF-α) acted synergistically to increase PAI-1 production. ET-1 activated protein kinase C and cAMP-dependent protein kinase pathways within 3 to 5 minutes of treatment, with the peak at 10 minutes. Activation of protein kinase C by phorbol-12-myristate-13-acetate (PMA) resulted in increased PAI-1 production, whereas activation of the cAMP-dependent protein kinase by forskolin or dibutyryl cAMP (dBu-cAMP) significantly decreased PAI-1 production. However, simultaneous activation of protein kinase C by PMA and cAMP-dependent protein kinase by dBu-cAMP only slightly attenuated PMA-induced PAI-1 increase. Inhibition of protein kinase C by GF-109213X abolished the effects of ET-1. These results demonstrate that ET-1 and TNF-α function synergistically to induce procoagulant activity of brain endothelial cells in a process that involves a protein kinase C-dependent pathway. (Arterioscler Thromb Vasc Biol. 1999;19:1768-1775.)

Key Words: endothelin • plasminogen activator inhibitor 1 • brain • endothelium • protein kinase C • cAMP

Increased levels of endothelin-1 (ET-1) in plasma or cerebrospinal fluid of patients with hypertension, ischemic stroke, and subarachnoid hemorrhage have implicated ET-1 as a mediator of cerebrovascular responses in these disorders.1–4 The endothelins (ETs) are a family of potent vasoactive peptides of 21 amino acids in length; cDNA cloning revealed 3 isopeptides, ET-1 to ET-3.5 ET has been shown to induce vasoconstriction,6 but has numerous other biological actions, including bronchoconstriction and hormone secretion,7–9 and acts on a variety of cell types, including endothelial cells (EC) and astrocytes.10–12 Two distinct ET receptor subtypes, ET-A and ET-B, have been cloned and sequenced.13,14 These receptors contain 7 hydrophobic transmembrane domains common to the superfamily of G-protein-coupled receptors; ET-A shows selective activity for ET-1 and ET-213 whereas ET-B shows nonselective affinity for all 3 isopeptides.14 EC possess predominantly ET-B receptors, which mediate vasodilation.15,16 It has been shown that the ET-A receptor is expressed on vascular smooth muscle cells and human brain EC, and mediates vasoconstriction.17,18 Both receptor subtypes are functionally coupled to phospholipase C via GTP-binding proteins in EC.19 Signal transduction on ET-1 binding to its receptor may differ among species and tissues and includes activation of phospholipase C and protein kinase C, mobilization of Ca2+ from intracellular stores, and influx of extracellular Ca2+, as well as inhibition of agonist-stimulated cAMP production.20–22

Clot formation and lysis is regulated, in part, by the potent proteolytic enzyme plasmin (see review in Reference 23). Inactive plasminogen is cleaved and converted to active plasmin by the enzyme tissue plasminogen activator (t-PA).24 The t-PA is rapidly inactivated by plasminogen activator inhibitor (PAI).25 Thus, net fibrinolytic activity in vivo is the result of the balance between the levels of t-PA and PAI.26,27 Of several PAI types, PAI-1 appears to be the most important in plasma.27 Serum PAI-1 levels have been found to be elevated in myocardial infarction,28–30 and are associated with an increased risk for thrombotic disease.31,32 EC produce t-PA, PAI-1, and PAI-2,33,34 which are regulated by factors such as thrombin, histamine, endotoxin, and cytokines, eg, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).35–39 These proinflammatory cytokines act directly on EC by stimulating procoagulation activity.20

In this study, human brain-derived EC were treated with ET-1 and examined for PAI-1 production. The data here demonstrate that ET-1 upregulates both PAI-1 mRNA and protein levels, as well as PAI-1 activity; and this PAI-1 induction is mediated by a protein kinase C (PK-C)-dependent pathway. Furthermore, TNF-α functions synergistically with ET-1 to increase PAI-1 production.
Methods

Reagents

Protein kinase A (PK-A) and PK-C assay kits were purchased from Gibco Life Technologies; cAMP assay kit from DuPont company; \(^{32}\)P\(\text{ATP}\) from ICN. Bisindolylmaleimide-GF-109203X (GF; PK-C inhibitor) was purchased from Calbiochem. Phorbol-12-myristate-13-acetate (PMA), dibutyryl cAMP (dBu-cAMP), and forskolin were obtained from Sigma. TNF-\(\alpha\) was purchased from Boehringer-Mannheim. ET-1 was obtained from Peninsula Laboratory.

Cell Culture

CNS EC were derived from human brain as previously described. Briefly, brain tissue, obtained after surgery, was digested with a series of collagenase/disperse treatments; cells were isolated on the basis of acetylated-LDL binding and FACS cell sorting. Purity of CNS EC was confirmed by immunocytochemical staining for factor VIII, glial fibrillary acidic protein, and the macrophage marker CD11b, as previously described; recovery routinely was >95% of CNS EC. Cells were cultured in RPMI-1640 medium (Gibco Labs), supplemented with 100 ng/mL endothelial cell growth factor Endogro (VECTEC), 2 mmol/L L-glutamine, 10 mmol/L HEPES, 24 mmol/L sodium bicarbonate, 300 U of heparin, 1% penicillin/streptomycin, and 10% FCS. Only cells from passage 4 to 5 were used in the described experiments; Endogro-free medium was used 24 hours before the initiation of experiments. The inhibitor GF (1 \(\mu\)mol/L) was added to cultures 30 minutes before ET-1 treatment.

PAI-1 Production

PAI-1 production was evaluated using the commercially available ELISA kit (American Diagnostics Corp). Briefly, cells were grown in culture to 60% to 70% confluence in 10% FCS. Culture supernatants (100 \(\mu\)L) were removed after 24, 48, and 72 hours and evaluated for PAI-1 content. The ELISA determined the total amount of bound and free PAI-1 present. The data for PAI-1 and t-PA are represented as the ratios of the concentrations of PAI-1 or t-PA protein derived from the supernatants of stimulated cell cultures compared with control cultures. The average control value for PAI-1 was 16.3 \pm 3.0 \(\mu\)g/mL per 10\(^6\) cells; the average control value for t-PA was 43.5 \pm 2.3 ng/mL per 10\(^6\) cells. Each experiment was performed 3 times, unless otherwise stated.

RNase Protection Assay

The radioactively labeled RNA antisense probe for PAI-1 was prepared using PCR; the \(\beta\)-actin template was provided by Ambion. Using the In Vitro Transcription Kit (Ambion), 6 \(\mu\)L of \(^{32}\)PTP (800 Ci/mmol, 10 mCi/mL; NEN Research) and 2 \(\mu\)L each of 10\(^\times\) transcription buffer, 17 polymerase, DTT, and RNasin were added as recommended by the manufacturer. The reaction was terminated by adding 1 \(\mu\)L RNase-free DNase for 1 hour at 37°C. The probes were then extracted using Tris-saturated phenol/chloroform/isooamyl alcohol (25:24:1) (GIBCO BRL) and chloroform/isooamyl alcohol sequentially, and then precipitated with ethanol. The radiolabeled RNA pellet was air dried and solubilized with hybridization buffer. RNA was isolated from 2 to 3 \times 10\(^5\) cells per experimental group and prepared according to a modification of the acid phenol method using the Trizol reagent (Life Technologies) as specified by the manufacturer. Total RNA (10 \(\mu\)g) together with 6 \times 10\(^5\) cpm of probe was heat denatured at 90°C and then hybridized overnight at 56°C. Subsequently, the samples were treated with the RNase cocktail, followed by proteinase K cocktails, then precipitated using ammonium acetate and ethanol. Air-dried samples were solubilized in 1X loading buffer, denatured at 90°C for 3 minutes, and then placed on ice. The protected fragments were resolved in 5% acrylamide 8 mol/L urea gel; the gel was dried and exposed to Hyper film (Amersham Life Science) at \(-70^\circ\)C. The protected bands were observed for PAI-1 (614 bp) and \(\beta\)-actin (320 bp). The differences among the groups were calculated as the ratio of the spectrophotometric densities of the experimental to control groups. The manufacturer’s recommended yeast tRNA negative control and a positive control were included in every RNase protection assay (RPA) experiment.

PAI-1 Activity Assay

The PAI-1 activity assay was performed using Pharmacia Coates PAI kit. A fixed amount of t-PA was added in excess to the conditioned media of the treated samples. A portion of t-PA forms an inactive complex with the PAI-1; subsequently, plasminogen was activated to plasmin by residual t-PA in the presence of the stimulator. The amount of plasmin formed is directly proportional to the PAI-1 activity in the conditioned medium. In the presence of chromogenic substrate, the color change was read at 405 nm.

cAMP Assay

The cAMP assays were performed using a kit (DuPont), according to the manufacturer’s instructions. Briefly, 2 to 3 \times 10\(^5\) EC were plated in media containing 10% FCS with no Endogro in 60-mm culture dishes 1 to 2 days before the experiment. The cells were grown to near (80% to 90%) confluence; 2 hours before initiating the experiment the growth medium was replaced with medium containing 1% FCS. The cells were treated with different agents for the specified time; the incubation was terminated by replacing the medium with 1 mL of 6% (wt/vol) trichloroacetic acid. The cells were then collected, homogenized with glass dounce homogenizer, and placed on ice for 30 minutes. The cell suspensions were centrifuged at 1500g for 15 minutes at 4°C. The supernatants were collected, and the trichloroacetic acid was removed by washing with water-saturated ether 3 times. Samples were lyophilized and redissolved in cAMP assay buffer (1 mL of sodium acetate, 0.6% EGTA, 0.2% sodium azide). A portion of the supernatant (100 \(\mu\)L) was acetylated to increase sensitivity. The cAMP content of the cells was subsequently measured by RIA (Rianen Assay System). The standard curve was obtained with unlabeled standard cAMP antigen (range, 0.1 to 4 pmol/mL) and a fixed amount of the \(^{32}\)I-labeled antigen reacted with a constant and limiting amount of antibody; a decreasing amount of the labeled antigen is bound to the antibody as the amount of unlabeled standard antigen is increased. These data were used to construct a standard curve from which the values in the samples were determined by interpolation. The data are expressed as mean \pm SD. All experiments presented here were performed at least 3 times unless otherwise stated.

PK-A Assay

EC were treated as described for the cAMP assays. The treatments were terminated by rinsing the cells with Dulbecco’s PBS and scraping them off with 0.5 mL of extraction buffer (5 mmol/L EDTA, 50 mmol/L Tris, pH 7.5). The cell suspensions were then homogenized and sedimented (10 000g, 10 minutes). The supernatants were collected for PK-A activity determination. The reaction was initiated by adding 10 \(\mu\)L of the lysates to 20 \(\mu\)L of reaction buffer; subsequently 10 \(\mu\)L of \(^{32}\)P\(\text{ATP}\) was added. The PK-A activity was determined as the difference between phosphorylation of a PK-A–specific substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly, kemptide) with or without a specific peptide PK-A inhibitor, which has an alanine to serine replacement in the consensus sequence Xaa-Arg-Xaa-Ser-Xaa and binds to the pseudosubstrate region of the regulatory domain of PK-A. The reaction was allowed to proceed at 30°C for 15 minutes and was terminated by removing a 20-\(\mu\)L aliquot to a phosphocellulose filter. Free \(^{32}\)P\(\text{ATP}\) was removed by washing the filter twice with both 1% phosphoric acid and water. Substrate phosphorylation was quantified by measuring the radioactivity on the phosphocellulose filters in scintillation fluid (Beta-fluor). All experiments were repeated at least 3 times and the data presented as mean \pm SD.

PK-C Assay

EC were treated as described in the previous sections. The PK-C assays were performed using PK-C assay kits obtained from Gibco. Briefly, the experimental treatments were terminated by replacing the medium with the cell extraction buffer (20 mmol/L Tris, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, and 25 \(\mu\)g/mL each aprotinin and leupeptin, pH 7.5) at room temperature. The cells were then homogenized with a precooled dounce homogenizer, and the cytosol and membrane fractions were separated by centrifugation (10 000g, 30 minutes, 4°C). The supernatants were collected as the
Results

ET-1 Upregulates PAI-1 Production by CNS EC
To test the hypothesis that ET-1 directly affects hemostasis, CNS EC were exposed to ET-1 at concentrations of 30 and 100 nmol/L for 72 hours and tested for PAI-1 production with ELISA. The results show that ET-1 significantly (P<0.02) increased PAI-1 production in a dose-dependent manner (Figure 1A). To determine whether the observed increase represented an upregulation in PAI-1 mRNA expression, RPA was performed. The results (Figure 1B) demonstrated that ET-1 upregulated PAI-1 mRNA by 80% after 1 hour of ET-1 treatment; this level was maintained at 3 and 6 hours, and then returned to baseline 24 hours after stimulation. To determine whether t-PA production was also altered by ET-1, culture supernatants were treated as described above and examined for t-PA with ELISA (Figure 1C). The results showed that t-PA was not affected by ET-1 at the optimal concentration used (100 nmol/L) (Figure 1C). The proinflammatory cytokine TNF-α, tested at an optimal concentration (10 ng/mL) alone or in the presence of ET-1, also did not alter the t-PA response.

To determine whether ET-1–treated CNS EC also exhibited an increase in PAI-1 activity, supernatant from 72-hour ET-1–treated cultures was examined for PAI-1 activity. The results in Figure 2 demonstrated that ET-1 increased PAI-1 activity by 63±7%, whereas TNF-α induced a 48±2% increase compared with untreated cultures (P<0.02). These data represent 1 of 3 similar experiments; each experiment was performed in triplicate.

Experiments were performed to determine whether TNF-α augmented the effects of ET-1 on PAI-1 production. The results in Figure 3 demonstrated that the addition of a suboptimal concentration of TNF-α (30 pg/mL) to a suboptimal dose of ET-1 (30 nmol/L) increased PAI-1 production by 92±16%, as compared with 35±12% with ET-1 alone. This level of PAI-1 production was comparable in magnitude with the maximal effects observed with optimal concentration of TNF-α (10 ng/mL) (Figure 3).

ET-1 Increases PK-A Activity in CNS EC
We next investigated which signal transduction mechanism was involved in ET-1–induced PAI-1 production. Treatment of CNS EC with ET-1 for 10 minutes resulted in a concentration-dependent increase in PK-A activity (Figure 4). The effect was significant at 10 nmol/L ET-1, with a maximal 5-fold activation reached at 100 nmol/L ET-1 (Figure 4). The time dependence of PK-A activity in the presence of 100 nmol/L ET-1 is given in Figure 5. A significant activation of PK-A by ET-1 was observed after 5 minutes of activation; activity reached a sharp maximum (5-fold activation) at 10 minutes and declined to the control levels after 20 minutes of incubation. Intracellular cAMP concentrations exhibited a similar time and dose response, reaching a maximum (0.767±0.047 fmol/10⁶ cells; mean±SEM) at 10 minutes for 100 nmol/L ET-1, with control values of intracellular cAMP...
at 0.327±0.031 fmol/10^6 cells (mean±SEM). The addition of forskolin and dBu-cAMP was routinely used as a positive control for both PK-A activity and cAMP elevation. These data demonstrate that ET-1, as with most reported PK-A activators, activates PK-A via increased intracellular cAMP concentrations.

ET-1 Increases PK-C Activity in CNS EC

The time course of the effect of 100 nmol/L ET-1 on membrane PK-C activity in CNS EC is shown in Figure 6. The increase in PK-C activity was significant at 2 minutes, reached a maximum at 10 minutes, sharply decreased by 20 minutes, and thereafter slowly declined to the longest observed time of 60 minutes, when the activity was still above the control levels (Figure 6). Because ET-1 activated both PK-A and PK-C in CNS EC, we used the cAMP-elevating agents forskolin or dBu-cAMP, or the PK-C-activating agent PMA to test the effects of activation of these signal transduction pathways on PAI-1 secretion. Figure 7A shows that both forskolin and dBu-cAMP dramatically decreased PAI-1 production, whereas activation of PK-C by PMA resulted in a 2.5-fold increase in PAI-1 production. A combination of both dBu-cAMP and PMA, although attenuating the effect of PMA alone, resulted in an approximately 2-fold increase in PAI-1 production relative to the control (Figure 7A), thus mimicking the effect of ET-1 on the CNS EC. The role of PK-C activation in ET-1–induced PAI-1 production was established by the use of the specific PK-C inhibitor, GF. CNS EC exposed to GF for 48 hours demonstrated a marked reduction in constitutive as well as ET-induced PAI-1 production (Figure 7B). Cells treated with GF for 48 hours were viable as measured by the trypan blue dye exclusion assay (>90%), and produced control levels of the chemokine IL-8 (data not shown), suggesting that the treated cells were alive and functional. These results demonstrated that ET-1 stimulation of PAI-1 is mediated through the PK-C–dependent signaling pathway.

Discussion

This work was initiated to examine the effect of ET-1 on the coagulation activity of human CNS EC, and to identify the intracellular signal transduction pathways responsible for this effect. The regulation of PAI-1 secretion by ET-1 appears to be a complex process, which will be best discussed by separating it into different parts, because there is no agreement in the literature on such important questions as the effect of ET-1 on PAI-1 or t-PA production, the effect of ET-1 on intracellular cAMP level, and the effect of increasing PAI-1 secretion by ET-1.
cAMP or PK-C activation on the production of PAI-1. First, there is no agreement in the literature on the effect of ET-1 on PAI-1 production. Previous studies of the effects of ET-1 on t-PA and PAI-1 production by EC failed to detect ET-1–induced increase in PAI-1 production. Yamamoto et al.42 found that ET-1 did not affect PAI-1 released from human umbilical vein endothelial cells (HUVEC), but suppressed thrombin-induced release of PAI-1 from HUVEC, and only at high concentrations of both thrombin and ET-1. Yamamoto et al.42 thus suggested that ET-1 may serve as an anti-fibrinolytic factor when EC-mediated fibrinolysis is stimulated by thrombin; they also suggested that both vasoconstriction and reduction of fibrinolysis are physiological roles of ET-1. This group also reported that although 100 nmol/L ET-1 did not affect PAI-1 secretion from HUVEC in 24 hours of incubation, it significantly attenuated the TNF-α–induced increase. A lack of effect of ET-1 (10 nmol/L, 24 hours) on basal t-PA or PAI-1 production by HUVEC was also reported by Rydholm et al.43 ET-1–induced PAI-1 increase was reported not in EC, but in rat mesangial cells by Iwamoto et al.44 This discrepancy may be because of the duration of the treatment with ET-1. In agreement with the results of Yamamoto et al.42 and Rydholm et al.43 we have also observed no significant increase of PAI-1 production induced by ET-1 in 24 hours; however a significant effect was achieved at the 48-hour time, with further increase relative to the control at 72 hours in the continuous presence of the peptide. Furthermore, we demonstrated an ET-1–induced increase in PAI-1 activity, which correlated with increased PAI-1 secretion, and no apparent effect on t-PA production.

Our results on the synergistic effect of low ET-1 and TNF-α concentrations to increase PAI-1 production by CNS EC are, however, in sharp disagreement with the observations of others42 who reported the attenuation of the PAI-1–increasing effect of TNF-α by ET-1 in HUVEC. One possible reason for the discrepancy between our results and those above may be because of the different types of EC used: Yamamoto et al.42 used HUVEC, systemic-derived macroves- sels, compared with CNS-derived microvascular cells, which are functionally different.45–47 For example, coagulation factors, eg, thrombin, stimulate EC from large vessels but not from the capillaries;48 elevation of glucose resulted in decreased PAI-1 mRNA in brain EC, but not in HUVEC;49 PMA suppressed the basic fibroblast growth factor-induced proliferation of capillary EC, but had no effect on aortic EC50; and vanadate treatment inhibited protein tyrosine kinase activity in aortic EC, but not capillary EC.51 Thus, these EC differ not only in the array of growth factor receptors present on their surfaces, but also in their intracellular regulatory mechanisms. The mechanism of the synergistic effects of TNF-α and ET-1 are not known, and may be attributed to several pathways. TNF-α may act indirectly through products of arachidonic acid metabolism or directly through intracellular enzymatic mediators (eg, protein tyrosine kinase). These processes are currently under investigation in this laboratory.

In the present work we found that ET-1 activates both PK-C- and cAMP-dependent signal transduction pathways. Again, there is no agreement in the literature regarding the effects of ET-1 on intracellular cAMP levels or PK-C activity. The effects of ET-1 on intracellular cAMP levels appear to be cell type-dependent, possibly related to coupling of ET₆ and ET₃ receptors to adenylyl cyclases via different G-proteins.52,53 Thus, ET-1 inhibits activator-induced cAMP increase in bovine EC,52 astrocytes,54 rat glioma cells,55 Swiss 3T3 cells,56 pig granulosa cells,57 and in rat nephron segments.58 ET-1–induced elevation of cAMP was observed in platelets.59 A small to negligible increase of cAMP by ET-1 was observed in rat glomerular mesangial cells,60 vascular smooth muscle cells,61 and intact preparations of rabbit aorta62 and atria.63 Ladoux and Frelin61 reported that in rat CNS EC, ET-1 inhibits cholera-toxin–induced increase in cAMP, whereas Stanimirovic et al.64 have shown that in human CNS EC, ET-1 increases intracellular cAMP. Inhibi-
tion of cAMP was previously associated with ET₃ receptors, and it was indeed thought that EC primarily possess this receptor type. Our results agree with those of Stanimirovic et al. and are consistent with the presence of ET₃ receptors on human CNS EC.

Most studies, including the present work, associate an increase in intracellular cAMP levels with a decrease in PAI-1 production. Studying the effect of intracellular cAMP levels on PAI-1 production, Slivka and Loskutoff showed that elevation of cAMP by forskolin and phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthene (IBMX) decreases PAI synthesis by bovine aortic EC. Rydholm et al. also found that forskolin decreases PAI-1 production by HUVEC to 61% of control, as compared with <40% of the control observed by us for the effect of forskolin or dBu-cAMP (Figure 7A). Santell and Levin reported that cAMP-elevating agents inhibited the PAI-1 synthesis in HUVEC. In contrast, only a small effect of forskolin and dBu-cAMP on PAI-1 production by HUVEC was found by Fukao et al. Thus, the elevation of cAMP by ET-1 or TNF-α observed in the present and previous studies cannot explain the increased PAI-1 production.

In addition to activation of PK-A, our data showed that ET-1 activated PK-C in CNS EC, agreeing with the results from other laboratories using various cell types. ET-1–induced release of inositol 1,4,5-triphosphate, which is an indication of the activity of phospholipase C, and generation of PK-C–activating diacylglycerol was reported in rat CNS EC by Stanimirovic et al. In both smooth muscle cells and fibroblasts, ET-1 rapidly stimulated a biphasic increase in diacylglycerol that was sustained for 20 minutes or longer. ET-1 stimulated rapid phosphoryldihydrochloride breakdown in Rat-1 fibroblasts and stimulated phospholipase C in Swiss 3T3 cells and in rat vascular smooth muscle cells leading to PK-C activation. ET-1–induced PK-C activation was also observed in rat aortic rings and in rat cardiomyocyte preparations.

Activation of PK-C by TNF-α was also demonstrated in several cell lines, including human EC cell line EA.hy 926, HUVEC, and human leukemic cell lines, agreeing with our observations on human CNS EC. In addition, direct measurements of PK-C activation of CNS EC at 10 minutes with TNF-α have demonstrated increased PK-C activity (Zidovetzki et al., unpublished observation, 1996). However, no effect of TNF-α on PK-C activity on HUVEC was reported by Ritchie et al. Mattila et al. explained the lack of PK-C activation by TNF-α reported in some studies by the observation that PK-C activity was measured at irrelevant times.

In a number of studies, activation of PK-C by PMA or other agents was associated with increased PAI-1 production in various EC, including HUVEC, bovine CNS EC, bovine aortic EC, and human CNS EC (this study). Thus, most of the observations in this and previous studies indicate that elevated cAMP is associated with a decrease, and activation of PK-C with an increase, in PAI-1 production. Moreover, Santell and Levin found that PMA-induced PAI-1 synthesis was prevented by elevation of cAMP. Nevertheless, Santell and Levin concluded that activation of cAMP in HUVEC, in coordination with PMA, led to a profibrogenic state in EC. A synergistic decrease of PAI-1 production by PMA and forskolin was reported by Sitter et al. Our results in CNS EC subjected to both PK-C activation by PMA and an increase in cAMP by dBu-cAMP partially agree with the results of Santell and Levin, and support their conclusion. In our system, dBu-cAMP attenuated PMA-induced PAI-1 production by CNS EC from 254±17% to 212±15%; thus the combination of both agents still resulted in a significantly higher PAI-1 production relative to the control (Figure 7A).

In conclusion, these findings acknowledge the role of ET-1 in regulating hemostasis activity in CNS EC; and further indicate that activation of PK-C is required for ET-1–induced increase in PAI-1 production by human CNS EC. With this information, the development of potent ET receptor antagonists or the use of specific signal pathway inhibitors may provide the tools necessary to study ET in cerebral ischemia and provide novel therapeutic approaches.

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References


66. Francis RB, Neely S. Inhibition of endothelial secretion of tissue-type plasminogen activator and its rapid inhibitor by agents which increase intracellular cyclic AMP. *Biochim Biophys Acta*. 1989;1012:207–213.


71. Wright HM, Malik KU. Prostacyclin synthesis elicited by endothelin-1 in rat aorta is mediated by an ET\(_A\) receptor via influx of calcium and is independent of protein kinase C. *Hypertension*. 1995;26:1035–1040.


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