Characterization of the Interactions Between Procoagulant Albumin and Human Endothelial Cells

By Kelly J. Faucette, Laurence A. Fitzgerald, Lihua Liu, Charles J. Parker, and George M. Rodgers

Normal human plasma contains procoagulant albumin (PC-AI), an anionic form of albumin that induces tissue factor (TF) activity in human umbilical vein endothelial cells (HUVEC) and monocytes. In this study, we investigated both the interactions between HUVEC and PC-AI and the mechanism by which PC-AI induces TF activity. Binding of PC-AI to HUVEC was specific and reversible. Further studies indicated that membrane-bound PC-AI was not internalized by HUVEC. A potential receptor on HUVEC was suggested by studies in which the capacity of a variety of reagents to inhibit the activity of PC-AI was quantitated. Induction of TF activity by PC-AI was antagonized by dextran sulfate, heparin, fucoidan, and concanavalin A but not by ovalbumin, polyglutamic acid, or polyvinyl sulfate. This competition profile bears similarities to those reported for scavenger receptors that have been identified on both HUVEC and monocytes. Involvement of protein kinase C (PKC) in the PC-AI-induced enhancement of TF activity was suggested by experiments in which staurosporine, an inhibitor of PKC, suppressed the activity of PC-AI. The induction of TF activity by PC-AI was further characterized by using a quantitative polymerase chain reaction assay. Increased TF mRNA was first seen after 1 hour of incubation with PC-AI. Maximal observed expression occurred at 2 hours, but at 5 hours, expression had significantly decreased. Monocytes could also be induced to express TF mRNA after a 2-hour incubation with PC-AI. These results suggest that the functionally relevant binding of PC-AI to HUVEC may be mediated through interactions with a membrane constituent that has some of the properties of a scavenger receptor and that this interaction augments TF activity by enhancing transcription of TF mRNA, at least in part, by a mechanism that is dependent on activation of PKC.

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EXPRESSiON of tissue factor (TF) activity by endothelial cells and monocytes appears to be a critical element in the process of thrombus formation at sites of vascular injury. Endothelial cells and monocytes do not express TF activity constitutively, but expression can be induced in these cells by factors (e.g., tumor necrosis factor [TNF], endotoxin, and interleukin-1 [IL-1]) that are generated in association with inflammatory processes. However, we have recently isolated a constituent of normal human plasma that induces TF activity in human umbilical vein endothelial cells (HUVEC) and monocytes. This factor was identified as a modified form of albumin and has been termed procoagulant albumin (PC-AI). Based on its behavior when chromatographed using an anion-exchange resin, the functionally active protein appears to be more negatively charged than the majority of plasma albumin (which is inactive). Inasmuch as PC-AI is present in normal plasma, we hypothesize that it is important in the regulation of hemostasis.

The purpose of this report is to characterize the interaction between PC-AI and HUVEC, to investigate the mechanism by which PC-AI induces TF activity in HUVEC and monocytes, and to present a polymerase chain reaction (PCR)-based approach to examine the time course and levels of TF mRNA production in PC-AI-treated HUVEC and monocytes.

MATERIALS AND METHODS

Tissue culture plasticware was obtained from Falcon Plastics (Oxnard, CA). Trizma base, ammonium sulfate, rabbit brain thromboplastin, reagents used for competition studies, buffers, and gel electrophoresis supplies were purchased from Sigma Chemical Co (St Louis, MO). Medium 199 (M199) and other tissue culture reagents were obtained from the University of California, San Francisco Cell Culture Facility. Phorbol 12-myristate 13-acetate (PMA) and staurosporine were supplied by Calbiochem (La Jolla, CA). Proplex-T concentrate was supplied by Baxter-Hyland (Glendale, CA). The chromogenic substrate S-2222 was purchased from Kabi Vitrum (Franklin, OH). 125I was obtained from Amersham Corp (Clearbrook, IL). Iodogen was purchased from Pierce Chemical Co (Rockford, IL). Native low-density lipoprotein (LDL) was kindly provided by Dr Robert E. Pitas (Gladstone Foundation Laboratories, San Francisco, CA). A silver-stain kit for identifying proteins in gels was obtained from Bio-Rad (Richmond, CA).

Tissue culture and protein purification methods. HUVEC were prepared as described and were used during the first passage. Assays were performed on cells plated in 24-well trays. Endothelial-cell TF activity induced by PC-AI was measured by using a chromogenic substrate assay. Optical density readings were converted to TF activity using a standard curve derived from results using a commercial thromboplastin preparation in which 1 μL of the thromboplastin stock is equivalent to 1,000 mU of TF activity. Purified human monocytes were obtained using an elutriation method. Purification of PC-AI was accomplished as previously described. The purified protein was homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; reducing conditions) and silver staining.

Binding studies. To assess binding to HUVEC, PC-AI was iodinated using the Iodogen technique by incubating 0.5 to 1.0 mg of protein at 4°C with 1 mCi of 125I in an Iodogen-coated Eppendorf tube (Eppendorf, Madison, WI). After 20 minutes, the radiolabeled...
PC-AI was separated from unincorporated iodine by using gel filtration chromatography. The specific activity of the radiolabeled protein ranged between 10 to 20 x 10^6 cpm/µg. A functional assay showed that the activity of radiolabeled PC-AI was equivalent to that of unlabeled PC-AI.

To assess the binding characteristics of PC-AI to HUVEC, M199 containing 0.5% bovine serum albumin and incremental concentrations of 125I-PC-AI or incremental concentrations of 125I-PC-Al and a 65-fold molar excess of unlabeled PC-AI were incubated at 4°C or 37°C with confluent HUVEC in 24-well trays. At timed intervals, the cells were washed 4 times with M199, and 100 µL of cell lysis buffer (2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L EDTA) were added. We found that 4 washes resulted in maximal specific binding of 125I-PC-AI to cells. Next, the solubilized cellular material was aspirated, and radioactivity was quantitated using a gamma counter.

Reversibility of binding was analyzed by incubating (at 4°C) 2 trays of HUVEC with M199 containing 8.6 µg/µL of 125I-PC-AL. After 30 minutes, a 65-fold molar excess of unlabeled PC-AI was added to 1 tray of cells, and the incubation was continued. At timed intervals, the cells were washed, and radioactivity was quantitated as described above.

Competition studies were performed by incubating HUVEC at 4°C with M199 containing either 125I-PC-AI (3.6 µg/µL) or 125I-PC-Al (3.6 µg/µL) and incremental concentrations of unlabeled PC-AI. After 30 minutes, the cells were washed, and radioactivity was quantitated as described above.

125I-PC-Al-saturation binding studies were performed using a modified Gauss-Newton-Marquardt-Levenber nonlinear least-squares procedure. The results of analysis are presented in terms of the mean parameter values ± estimates of standard errors obtained from the analysis. Data were analyzed with models of increasing complexity. The model that was chosen for reporting the results is that which yielded the smallest sum of squares and represented a significant improvement over simpler models. Thus, in the best model, the experimental data were fitted by the Hill function:

\[ B = B_{\text{max}} \cdot \frac{(F/K)^n}{1 + (F/K)^n} + B_0 \]

where B represents the extent of ligand binding, B_{max} represents maximal ligand binding capacity, K represents the dissociation constant, F represents free ligand concentration, n represents the Hill coefficient, and B_0 represents baseline binding.

To determine if HUVEC internalize PC-AI, cells were incubated at both 4°C and 37°C with 125I-PC-Al. At timed intervals the cells were washed. One set of cells (the controls) was immediately solubilized. A second set of cells was incubated for 4 minutes at 37°C with buffer containing 25 µg of trypsin. This treatment caused the cells to dissociate from the tissue culture plate. The cell suspension was aspirated from the wells of the tissue culture plate and centrifuged (10 minutes at 10,000 g). The supernate was aspirated, and the radioactivity of the supernate, the cell pellet, and the solubilized cells was subsequently quantitated.

Two types of competition studies were performed. First, the capacity of a variety of reagents to inhibit the functional activity of PC-AI was investigated. HUVEC were incubated at 37°C with buffer containing a constant amount of PC-AI (10 to 100 µg/mL) either alone or with incremental concentrations (0.1 to 1,000 µg/mL) of the test reagent. After 4 hours, the cells were washed, and TF activity was determined using a chromogenic substrate assay. Second, the capacity of a variety of reagents to inhibit the binding of 125I-PC-AI was investigated. HUVEC were incubated at 37°C with buffer containing a constant amount of 125I-PC-Al (5 to 10 µg/mL) either alone or with 300 µg/mL of the test reagent. After 30 minutes, the cells were washed and solubilized, and radioactivity was subsequently quantitated.

Evaluation of the protein kinase C (PKC) mechanism in TF induction. To determine if PKC participates in the process by which PC-AI induces TF activity, HUVEC were incubated at 37°C with buffer containing either PC-AI (100 to 200 µg/mL) or PC-Al (100 to 200 µg/mL) and staurosporine (50 to 100 nmol/L). After 4 hours, TF activity was quantitated by using the chromogenic substrate assay. For controls, HUVEC were incubated with buffer, with buffer containing staurosporine (100 nmol/L) with PC-Al (100 µg/mL) and dimethyl sulfoxide (DMSO; at the same concentration as that in the samples containing staurosporine). As an additional control, HUVEC were incubated with buffer containing 100 nmol/L PMA or with buffer containing 100 nmol/L PMA and 100 nmol/L staurosporine.

TF mRNA studies. The capacity of PC-AI to induce TF mRNA production was also assessed by a quantitative polymerase chain reaction (PCR) technique. For these studies, control fibroblasts, HUVEC, and freshly isolated monocytes were compared with HUVEC and monocytes treated with PC-AI for 1 to 5 hours. The treated and control cells (10^6 to 10^7) were washed once in serum-free media, frozen at -70°C, and, at a later time, total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi using a kit from Stratagene (La Jolla, CA). The purified RNA had an A260/A280 ratio of greater than 2.0, and 18S and 28S ribosomal RNA bands appeared undecorated when analyzed on formaldehyde-agarose gels (not shown).

To produce a PCR template, single-strand cDNA was synthesized using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (200 U; Bethesda Research Laboratories, Gaithersburg, MD) on 1 µg of total RNA using standard protocols and reagents supplied with the enzyme. Equivalent apparent results for PCR amplification were obtained with cDNA synthesis primed using either random 9-mer oligonucleotides or oligo(dT)14-20.

To detect the presence of TF, a PCR-primer set was synthesized based on the cDNA sequence of Morrissey et al to give an expected product of 310 bp. To eliminate potential contribution of genomic DNA, the primers were located on exons 2 and 4 of the published TF gene. TF forward primer, 5'-GAC AAT TTT GGA GTG GGA ACC C 3' (cDNA no. 189 through 210); TF reverse primer, 5'-CAC TTG TCG CAC CAC TCC C 3' (cDNA no. 498 through 481).

To normalize the PCR amplification in different cDNA preparations, a second set of PCR oligonucleotide primers against a control protein, glyceraldehyde-3-phosphate dehydrogenase (GPDH), was synthesized as described by Hla and Maciag. GPDH was produced as described by Hla and Maciag. GPDH forward primer, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; GPDH reverse primer, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. The GPDH primers produce an expected product of 650 bp and are also on separate exons.

A Hybaid thermal cycler (Model HB, TR-1) was used for 22 to 28 cycles, with cycle conditions of 90 seconds each at 95°C, 55°C, and 73°C using the plate mode to regulate temperature. PCR reactions for each cDNA were set up in 300 µL volumes, then 100 µL was aliquotted into 3 separate reaction tubes. Taq polymerase (2.5 U; Boehringer Mannheim, Indianapolis, IN) was added after the tubes had warmed to greater than 80°C. The reaction tubes contained 0.15 µg of each GPDH primer and 0.5 µg of each TF primer, 1 µL of a 25 nmol/L solution of each dNTP (Pharmacia), and 1.5 reaction buffer with 1.5 mmol/L Mg²⁺ from Boehringer Mannheim. The cDNA templates were added such that 1% to 4% of the total cDNA synthesis mix was added per PCR reaction. To label the TF and GPDH PCR products for quantitation, 1 µCi of α-[32P]dCTP (Amersham) was added per 100 µL reaction volume to give a final
concentration of 1.16 pmol/L. Reaction tubes were removed at different cycle number, and equal aliquots were fractionated on 1.3% agarose/TBE gels and stained with ethidium bromide. To quantitate the amount of specific TF and GPDH products, the GPDH (630 bp) and TF (310 bp) bands were excised and counted in a Beckman scintillation counter (Beckman Instruments, Irvine, CA). We quantitated the amount of TF product as the ratio of TF dpm:GPDH dpm. This ratio from HUVEC and monocyte samples was then compared with that from fibroblasts to determine the fold increase in mRNA levels.

RESULTS

$^{125}$I-PC-AI binding studies. To demonstrate that $^{125}$I-PC-A1 and unlabeled PC-A1 bind equally well to endothelial cells, varying ratios of $^{125}$I-PC-A1/PC-A1 were incubated with the cells, and the amount of $^{125}$I-PC-A1 bound to the cells was plotted against the percent $^{125}$I-PC-A1 in each sample. In this experiment, bound cpm decreased by 52% when the percentage of $^{125}$I-PC-A1 was reduced by 50%. A straight line ($r = .945$) was obtained (data not shown). These data indicate that native and radiolabeled PC-A1 bind identically to these cells. Our next studies were designed to characterize the binding of PC-A1 to HUVEC. At 4°C, the binding isotherm developed a plateau phase after approximately 20 minutes, with maximum binding occurring after approximately 30 minutes (Fig 1A). At 37°C, maximum binding was achieved within 5 minutes and remained constant over 45 minutes of observation (not shown). When a 65-fold molar excess of unlabeled PC-A1 was added to samples that had been incubated with $^{125}$I-PC-A1 for 30 minutes at 4°C, binding of the radiolabeled protein was reduced by approximately 50% within 5 minutes, suggesting that binding of PC-A1 is reversible (Fig 1A). The addition of incremental amounts of unlabeled PC-A1 to a fixed amount of the radiolabeled protein caused a concentration-dependent decrease in binding of $^{125}$I-PC-A1 (Fig 1B). The presence of a 129-fold excess of unlabeled PC-A1 resulted in a 70% reduction in binding of the labeled protein. These results, combined with competition studies discussed below, suggest that binding of PC-A1 to HUVEC is specific.

A saturation binding study was conducted using concentrations of $^{125}$I-PC-A1 ranging from 0.05 to 100 µg/mL. Figure 2 depicts these results as plotted with a nonlinear least-squares computer program to yield the best-fit model. The inset to Fig 2 shows the slow rate of rise of the binding curve, suggesting positive cooperativity. Values for $K$ and $B_{max}$ are 22.5 ± 1.9 µg/mL and 32.3 ± 1.7 ng/well bound $^{125}$I-PC-A1, respectively. Conversion of the $B_{max}$ value to molecules of PC-A1 per cell indicates that $3.4 \times 10^{10} \pm 1.8 \times 10^{9}$ molecules of $^{125}$I-PC-A1 bind to HUVEC.

To determine if PC-A1 is internalized, HUVEC that had been incubated at either 4°C (Fig 3A) or 37°C (Fig 3B) with $^{125}$I-PC-A1 were washed and treated with trypsin. Trypsinization caused the cells to detach from the wells of the tissue culture plate. After centrifugation, the radioactivity associated with the supernate and cell pellet was quantitated. The radioactivity associated with cells that had not been trypsinized was also quantitated in parallel. After treatment with trypsin, only a small amount of radioactivity remained associated with the HUVEC, and there was no distinct increase in cell-associated radioactivity after 50 minutes of observation (Figs 3A and 3B). The radioactivity in the supernate of the trypsin-treated cells was nearly the same as the radioactivity associated with untrypsinized cells. These results suggest that PC-A1 is not internalized by HUVEC in vitro.
Our previous results suggested that a scavenger-like receptor might mediate the effects of PC-Al, because dextran sulfate, an inhibitor of both the acetyl-LDL receptor\textsuperscript{16} and the putative scavenger receptors on rat endothelial cells,\textsuperscript{17,18} prevented induction of TF activity by PC-Al.\textsuperscript{4} A representative experiment in which an effective competitor (heparin) and an ineffective competitor (ferritin) were assayed for the capacity to inhibit PC-Al-induced TF activity in HUVEC is shown in Fig 4. Heparin (Fig 4A) exerted a concentration-dependent inhibition of the PC-Al-induced TF response, whereas ferritin (Fig 4B) had no inhibitory activity. Table 1 summarizes the results of competition experiments using a variety of reagents. A result was termed negative if there was less than 25% inhibition when the test reagent was used in a 50-fold (wt/wt) excess of PC-Al. A result was called positive if the reagent inhibited the activity of PC-Al by greater than 50%. The ratio of dextran sulfate and fucoidan:PC-Al that caused greater than 50% inhibition was 0.5 to 1.0:1; for heparin and concanavalin A, the ratio was 1 to 2:1.

The results of studies on the effects of the various reagents on PC-Al-induced enhancement of TF expression are compared with previously published studies of effective and ineffective competitors of the acetyl-LDL receptor\textsuperscript{16} and of the rat endothelial cell scavenger receptors.\textsuperscript{17} In 5 of 8 instances in which results could be compared, the effects on the acetyl-LDL receptor were the same as for the putative PC-Al receptor (Table 1). Compared with the rat endothelial cell scavenger receptors, the effects on PC-Al were equivalent in 7 of 8 cases. When the effects on the acetyl-LDL receptor were compared with the effects on the rat endothelial cell scavenger receptor, 4 of 6 comparable reagents produced similar results. These studies suggest that the HU-
VEC-binding site for PC-AI may share some properties with scavenger-like receptors.

The effects of various reagents on 125I–PC-AI binding to HUVEC were correlated with the effects of these reagents on inhibition of PC-AI–induced TF activity. As shown in Fig 4C, dextran, heparin, fucoidan, and concanavalin A, but not ovalbumin and ferritin, inhibited binding of 125I–PC-AI. Similar results were seen when these reagents were tested for their ability to induce TF activity (Table 1).

Role of the PKC mechanism in TF induction. To determine if PKC participates in the process whereby PC-AI induces TF activity, the effects of staurosporine, an inhibitor of PKC, on TF expression by HUVEC treated with PC-AI were studied. As previously reported by others, PMA induced TF in HUVEC, and this process was inhibited by staurosporine (Fig 5). The effects of PC-AI on TF expression by HUVEC were in the same range as those for PMA (Fig 5), and staurosporine significantly inhibited the activity of PC-AI (Fig 5). In 4 separate experiments, each with triplicate determinations, staurosporine inhibited PC-AI activity by 50% to 87%. In parallel experiments, staurosporine did not inhibit the binding of 125I–PC-AI to endothelial cells (data not shown). These results suggest that the procoagulant activity of PC-AI may be mediated, at least in part, by a PKC-dependent mechanism.

PC-AI induces TF mRNA. Our earlier studies showed that maximal induction of TF expression by PC-AI required between 2 and 4 hours. This time course is consistent with the hypothesis that binding of PC-AI to HUVEC resulted in de novo synthesis of TF by stimulating transcription of TF mRNA. To confirm this hypothesis, the effects of PC-AI on
The purpose of the present study was to investigate the interactions of PC-AI with HUVEC and to characterize the mechanism by which PC-AI induces TF expression. Binding of PC-AI to HUVEC appears to be specific, saturable, and reversible (Figs 1 and 2). Schnitzer et al. analyzed the

![Bar chart with data points and error bars showing the effect of PKC inhibitor staurosporine on TF activity.](chart-url)

**Fig 6.** Effect of the PKC inhibitor, staurosporine, on PC-AI-induced TF activity. HUVEC were incubated at 37°C with buffer, with buffer containing staurosporine (100 µmol/L) with buffer containing PC-AI (100 µg/mL) and DMSO (the solvent for staurosporine), with buffer containing PC-AI (100 µg/mL) and staurosporine (100 µmol/L), with buffer containing PMA (100 nmol/L), or with buffer containing PMA (100 nmol/L) and staurosporine (100 µmol/L). After 4 hours, TF activity was quantitated. The bars depict the mean ± SEM (n = 3). *Samples with values statistically different from control (P < .05.) Staurosporine inhibits the functional activity of PC-AI.
binding of rat albumin to endothelial cells derived from rat epididymal fat pads. They reported that binding was specific, saturable, and reversible and that equilibrium was reached in approximately 20 minutes at 4°C. Analysis of their data by Scatchard’s method generated a curvilinear plot that was consistent with either negative cooperativity or with the presence of multiple binding sites. Our results suggest positive cooperative binding for PC-AI on HUVEC (Fig 2). Positive cooperativity may explain the discordance seen between the dissociation constant estimated by direct binding (22.5 μg/mL) and competition binding (>100 μg/mL).

Studies by others suggest that rat endothelial cells express more than one type of receptor for albumin. A sialoglycoprotein with a molecular weight (Mₚ) of 60 kD (gp60) that binds native albumin has been identified. This protein that shares antigenic epitopes with glycophorin (the major erythrocyte sialoglycoprotein) is expressed by continuous endothelium, binds native albumin, and appears to be involved in albumin transcytosis. Albumin-binding proteins with Mₚ's of approximately 30 kD (gp30) and 18 kD (gp18) have also been identified. These proteins have a different tissue distribution compared with gp60, and they appear to have a higher affinity for modified albumin than for native albumin. Further characterization of their binding properties has suggested that gp30 or gp18 are scavenger-like receptors. Whether the human analogue of gp30 and/or gp18 is the receptor for PC-AI remains to be determined.

One of the features of ligands recognized by scavenger receptors is that they contain a region with a high density of negatively charged residues. Thus, a modification that endows a protein with a highly anionic region may cause it to be recognized by scavenger receptors. Although the modification that engenders albumin with the capacity to induce TF expression has not been identified, PC-AI is distinguished from the majority of plasma albumin (which is inactive) by a difference in charge, with PC-AI showing increased affinity for a positively charged chromatographic matrix. Accordingly, we postulated that binding of PC-AI to HUVEC might be mediated by a scavenger receptor. In support of this hypothesis, heparin sulfate, a molecule with a strong negative charge, inhibited the activity of PC-AI in a concentration-dependent fashion (Fig 3A), whereas ferritin (isoelectric point = 5.0) had no effect (Fig 3B). Studies by others have defined a competition profile for the acetyl-
LDL scavenger receptor and for the rat endothelial cell scavenger receptors. The competition profile for PC-AI overlapped with that of both the acetyl-LDL scavenger receptor and the rat endothelial cell scavenger receptors (Table 1). Thus, although definitive proof is lacking, it seems plausible to suggest that binding of PC-AI to HUVEC is mediated in part by a scavenger-like receptor and that a consequence of this receptor-ligand interaction is transduction of the signal that causes enhancement of TF expression.

Trypsinization released essentially all of the 125I–PC-AI that was bound to HUVEC (Fig 3). These results indicate that PC-AI is not internalized and suggest that signal transduction is mediated (directly or indirectly) as a consequence of interactions with a receptor that is expressed on the surface of HUVEC. These results also imply that PC-AI does not enhance TF-gene transcription directly by functioning as a trans-acting factor. The failure to observe internalization also suggests that the putative receptor for PC-AI does not show all of the properties of a scavenger receptor, because the primary purpose of scavenger receptors is to provide a mechanism whereby proteins that are targeted for removal can be endocytosed and degraded. The mechanism whereby trypsin releases PC-AI from HUVEC is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious. The rapidity and completeness of the process suggest that the HUVEC-binding site for PC-AI is not obvious.

Studies by others indicate that the mechanism whereby IL-1, TNF, and endotoxin enhance expression of TF by HUVEC on TF expression is dependent on activation of PKC. Our studies suggest that the effects of PC-AI are also, at least in part, PKC-dependent (Fig 5). Thus, activation of PKC appears to be a common mechanism whereby procoagulant stimuli induce expression of TF activity.

Our previous studies have shown that an incubation period of between 2 and 4 hours is required for the effects of PC-AI on HUVEC to become apparent, that maximal enhancement of TF expression is observed between 4 and 6 hours, and that expression declines thereafter. These observations are consistent with the hypothesis that PC-AI stimulates de novo synthesis of TF. To test this hypothesis, we developed a quantitative PCR assay to quantitate and characterize the time course of TF-mRNA induction by PC-AI. The advantages of this method are its sensitivity and the need for relatively few numbers of cells to prepare adequate amounts of RNA and cDNA for amplification. Thus, we were able to repeat the experiments several times to assure reproducibility of the results shown in Figs 6 and 7. The variability inherent in the PCR methods were controlled in the following ways: (1) preparation of all total RNA at the same time, using identical reagents and cell isolation techniques; (2) preparation of cDNA with identical amounts of input RNA, primers, and reagents; and (3) use of a control set of amplification primers based on the constitutive enzyme GPDH that is unlikely to vary greatly among the different cell types or under conditions of HUVEC stimulation with PC-AI. We observed a very consistent amount of the GPDH-specific PCR product among the cell types used; the amount was such that only minor adjustments in cDNA input were needed to normalize the GPDH product. For all amplifications using HUVEC cells, the cDNA input was identical. We performed PCR with the primer sets used separately and in multiplex reactions, and the results were very similar (Fig 6).

We decided to present data using both methods, because, in the multiplex mode, there was minor attenuation of the TF primer-specific product. This artifact affected the results most significantly with the fibroblasts and the PC-AI–treated monocytes.

The most surprising result in the PCR studies was the magnitude of the apparent TF mRNA induction in the HUVEC compared with fibroblasts. TF PCR product was not detected in control RNA/cDNA preparations from HUVEC and monocytes; thus, the most reasonable expression of TF mRNA levels in the PC-AI-induced HUVEC and monocytes was relative to fibroblasts. In the experiments shown in Figs 6 and 7, we calculated an approximately fivefold greater amount of TF mRNA in the 2-hour treated HUVEC relative to normal fibroblasts. Thus, there is a rapid but short-lived induction of TF mRNA by PC-AI that is similar to that reported using Northern hybridization analysis of THP-1 monocytic cell RNA after induction with endotoxin.

Incubation of both HUVEC and monocytes with PC-AI enhanced expression of TF mRNA. Inasmuch as neither HUVEC nor monocytes express TF mRNA constitutively, these results indicate that the effects of PC-AI up to 2 hours postinduction are mediated through a process that enhances...
TF gene transcription. Studies by Mackman et al72 have suggested that the promoter region of the TF gene contains a serum-responsive element. The inhibitory effects of staurosporin (Fig 5) suggest that the effects of PC-AI are mediated by a trans-acting factor (eg, AP-1) that is activated by PKC.

The functional significance of PC-AI remains to be established. However, our findings that a constituent of normal plasma has the capacity to induce TF expression in both endothelial cells and monocytes suggest that PC-AI may be involved in both physiologic and pathophysiologic events related to the processes of hemostasis and thrombosis.

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REFERENCES

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KJ Faucette, LA Fitzgerald, L Liu, CJ Parker and GM Rodgers