Induction of Angiotensin I-converting Enzyme Transcription by a Protein Kinase C-dependent Mechanism in Human Endothelial Cells*

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Angiotensin I-converting enzyme (ACE)1 has been implicated in various cardiovascular diseases; however, little is known about the ACE gene regulation in endothelial cells. We have investigated the effect of the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) on ACE activity and gene expression in human umbilical vein endothelial cells (HUVEC). Our results showed a 3- and 5-fold increase in ACE activity in the medium and in the cells, respectively, after 24-h stimulation by PMA. We also observed an increase in the cellular ACE mRNA content starting after 6 h and reaching a 10-fold increase at 24 h in response to 100 ng/ml PMA as measured by ribonuclease protection assay. This effect was mediated by an increased transcription of the ACE gene as demonstrated by nuclear run-on experiments and nearly abolished by the specific PKC inhibitor GF 109203X. Our results indicate that PMA-activated PKC strongly increases ACE mRNA level and ACE gene transcription in HUVEC, an effect associated with an increased ACE secretion. A role for early growth response factor-1 (Egr-1) as a factor regulating ACE gene expression is suggested by both the presence of an Egr-1-responsive element in the proximal portion of the ACE promoter and the kinetics of the Egr-1 mRNA increase in HUVEC treated with PMA.

ACE is a membrane-bound enzyme located mainly at the luminal face of endothelial cells (EC) in blood vessels (2). In the plasma, ACE is present as a soluble form originating mainly from EC by a proteolytic cleavage (3).

The level of ACE is stable in human plasma, and limited data are available on its regulation at the cellular or tissue level (4). In bovine aortic endothelial cells, ACE was shown to be increased slightly by glucocorticoids, density growth arrest, and ACE inhibitors (5–8). Glucocorticoids also induce ACE expression and activity in macrophages and rat vascular smooth muscle cells (9, 10). In the latter cells, the induction is synergistic with basic fibroblast growth factor (11). ACE induction is also observed in several pathological processes. After balloon injury in rat arteries, ACE is induced in vascular neonatal cells, and ACE inhibition has a beneficial effect on neointimal proliferation in this experimental model, decreasing the neointima/media ratio (12). ACE was shown to be induced in the aortic wall in different models of rat hypertension such as renovascular hypertension (13) or hypertension induced by chronic administration of the nitric oxide synthesis inhibitor L-nitro-arginine-methyl ester (14). ACE expression is also increased in interstitial cells of the heart during renovascular hypertension (15).

Whether ACE induction plays an etiological role in pathological processes or simply reflects an endothelium activation is still a matter of debate. In the rat, ACE cDNA transfer in the vessel wall leads to an increased media/lumen ratio without systemic effects (16). In the N'2-nitro-L-arginine-methyl ester model of hypertension, non-antihypertensive doses of ACE inhibitors seem to inhibit coronary vessel and cardiac remodeling (14).

The importance of ACE in cardiovascular diseases also comes from the possible implication of a polymorphism of the human ACE gene, which is associated with increased plasma and cellular ACE levels (17–19). The deletion allele of the insertion/deletion marker of the ACE gene was shown to be associated with an increased risk for myocardial infarction, left ventricular hypertrophy, vascular wall thickness, or vascular complications of diabetes (20). Results of these studies are not all concordant as can be expected from the modest relative risk increase that was found to be associated with the ACE genotype.

In various conditions, such as diabetes, platelet-activating factor, or basic fibroblast growth factor stimulation, an increase in ACE activity has been reported in vivo or in vitro in vascular smooth muscle cells and EC (11, 21–24). In these same experimental conditions, activation of PKC has also been reported in these cells (25–27). To determine more precisely how the ACE gene is regulated in EC, we investigated the effect of PMA-activated PKC on ACE gene expression and on ACE secretion. Our results indicate that PMA strongly increases

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1 The abbreviations used are: ACE, angiotensin I-converting enzyme; EC, endothelial cells; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Egr-1, early growth response factor-1; HUVEC, human umbilical endothelial cells; GF 109203X, bisindolylmaleimide I hydrochloride; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; RT, reverse transcription; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid.
ACE mRNA levels and ACE gene transcription in EC, an effect associated with an increased ACE secretion. A possible mechan- anism of ACE transcription induction is suggested by the presence of an Egr-1 responsive element in the ACE gene promoter and by the kinetics of Egr-1 mRNA increase by PMA in HUVEC, which is compatible with such an effect.

EXPERIMENTAL PROCEDURES

Materials—Collagenase H, sonicated salmon sperm DNA, DNaI, proteinase K, RNase A, and T1 were from Boehringer Mannheim (Mey- lan, France). Phorbol 12-myristate 13-acetate (PMA), MCDB-131 me- dium, EDTA, and heparin sodium heparinized 1 hydroxy-corticosterone (GF 109203X) were from Sigma (L’Isle d’Abeau Chesnes, France). Fetal calf serum, phosphate-buffered saline, 1-glutamine, penicillin, and strepto- mycin were from Seromed (Berlin, Germany). Human recombinant epidermal growth factor was from Euromedex (Souffleweyersheim, France).

Cell Culture—HUVEC were isolated as described in detail by Jaffe et al. (28). The vein of an umbilical cord was washed with 20 ml of washing buffer (140 mM NaCl, 0.15 mM KH2PO4, 4 mM KCl, 0.5 mM NaH2PO4, and 2% glucose (w/v)). EC were dissociated from the vessel wall with 2% (w/v) collagenase H in washing buffer for 10 min at 37 °C. Cells were grown to confluence in a culture medium containing MCDB 131 me- dium, 10% (v/v) fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml human recombi- nant epidermal growth factor, and 1 μg/ml hydrocortisone. Cells were maintained at 37 °C, 5% CO2 in a humidified incubator. Cells were labeled by staining for factor VIII- (Immunotech, Marseille, France) and CD31- (DAKO, Carpinteria, CA) related antigens. Cells were used at the first passage only. For all experiments, 24-h confluent cells were depleted of serum (0.4%) and of hydrocortisone/human recombinant epidermal growth factor for 24 h before PMA treatment. PMA and the PKC inhibitor GF 109203X stock solutions were made in dimethyl sulfoxide. In each experiment, the vehicle concentration was kept constant regardless of differences in the concentration of the compounds tested for PMA inhibition, GF 109203X was added 1 h before a 24-h PMA treatment. Messenger RNAs were then harvested for ribonuclease protection assay.

ACE Activity Assay—Control or PMA-treated cells from individual 25-cm2 plates were scraped after two washes in ice-cold phosphate-buffered saline. Cells and culture medium were stored frozen until assayed for ACE activity.

Cellular ACE Activity—Cells in phosphate-buffered saline were pel- leted and resuspended in 500 μl of 50 mM NaCl, 4 mM KCl, 10 mM CsCl, and 8 mM CHAPS; then they were sonicated and centrifuged. ACE activity was determined essentially as described previously (29). Briefly, 100 μl of supernatant was incubated for 30 min at 37 °C with 25 μmol of the ACE substrate Z-Phe-His-Leu, and the produced dipeptide His-Leu was measured on a spectrofluorometer (Hitachi F2000) after labeling to the fluorescein 1,2-phthallicdicarboxaldehyde.

Medium ACE Activity—ACE activity in the medium was measured on the radiolabeled synthetic substrate [glycine-1-14C]Chippuryl-histi- dine-leucine as described previously (30). Activities were standardized to the total cellular protein content determined by the Bradford method (31).

Ribonuclease Protection Assay (RPA)—Riboprobes were labeled with [α-32P]UTP (800 Ci/mmol, Amersham Pharmacia Biotech) using a commercially available kit (Riboprobe system, Promega) and T3 RNA poly- merase. The RPA probe for ACE was obtained by NoI digestion of a plasmid (pBS-KS, Stratagene) containing the complete human ACE cDNA into its EcoRI site. This linearized template generates a ribo- probe of 345 nucleotides and an expected protected ACE fragment of 280 bp (nucleotides 3742–4022 of the cDNA). For the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, we cloned into pCR-Script (Stratagene) a RT-PCR product from nucleotide 671 to 1110. This clone was digested with Bsa I leading to a full-length probe of 270 nucleotides and a protected fragment of 210 bp. Total cellular mRNA were isolated from HUVEC using commercially available reagents (Trizol, Life Tech- nologies) based on a published method (32). Determination of the RNA concentration was achieved by spectrophotometry measuring A260 nm. RNA quality was evaluated by the 260/280 nm A ratio and their quality by agarose gel electrophoresis and ethidium bromide staining.

RT-PCR—RT reactions were performed with 1 μg of RNA, 10 pmol of oligo(dT)12-18 (Amersham Pharmacia Biotech), 200 units of Moloney murine leukemia virus reverse transcriptase (Life technologies), 40 units of ribonuclease inhibitor (RNaseOUT, Life Technologies), and 1 μg of each concentration of each dNTP in a total volume of 20 μl of standard RT buffer. Samples were incubated for 1 h at 37 °C, and the reaction was stopped by heating for 10 min at 80 °C.

GAPDH and Egr-1 DNA amplifications were performed in the same PCR to allow a relative quantification of Egr-1 mRNA. Touchdown PCR was performed as follows. First, 1 μl of cDNA was amplified with 10 pmol of 5’- and 3’-Egr-1 primers, 1 unit of Taq polymerase (Life Technologies), a 0.1 mM concentration of each dNTP, and 1 μCi of [α-32P]dATP (2500 Ci/mmol, Amersham Pharmacia Biotech) in a total volume of 50 μl of PCR polymerase buffer. For each PCR, the following conditions, each step lasting 30 s: 3 cycles at 68, 72, and 95 °C at 66, 72, and 95 °C with Egr-1 primers. After the addition of 10 pmol of 5’- and 3’-GAPDH primers, 20 cycles were performed at 65, 72, and 95 °C. 10 μl of each amplification mixture was electrophoresed on 1.5% agarose gels. Expected size products were 480 bp for GAPDH and 253 bp for Egr-1. Primer sequences were as follows: GAPDH, 5’-CATATCGTCTGCTTGG-TCCAG-3’ (forward) and 5’-GCTTCGCTTCTGGTCCG-3’ (reverse); Egr-1, 5’-CTTCTGCTGAGGCACCAT-3’ (forward) and 5’-CAGACACTGTTGAGCTGCA-3’ (reverse). Gels were dried before quantification of radioactive signals.

Statistics—Student’s t test was used for comparison of ACE activities and run-on results, and significance was accepted for p < 0.05. For dose response, PKC inhibition, kinetics, and Egr-1 mRNA induction analy- sis, a repeated measures one-way analysis of variance was used. The p value was calculated by the Fisher test, and significance accepted for p < 0.05.
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RESULTS

ACE activity was measured in HUVEC extracts and culture medium after a 24-h PMA treatment. As shown in Fig. 1, cellular ACE activity was increased 5-fold (70 ± 20 versus 13 ± 8 pmol of Z-Phe-His-Leu/μg/min) after PMA treatment. Similarly, there was a 3-fold increase in ACE activity detected in the culture medium (4.2 ± 1.8 versus 1.5 ± 0.7 pmol of His-His-Leu/μg/min).

An RPA was used to investigate if the increase in ACE activity was associated with an increase in the mRNA level. ACE mRNA was measured together with the GAPDH mRNA as an internal control for the amount of RNA hybridized. We looked at the kinetics of ACE mRNA induction. Cells were incubated in the presence or the absence of PMA (100 ng/ml) for different periods (from 0 to 48 h), and mRNAs were collected and analyzed by RPA. As shown in Fig. 2, the ACE mRNA increases after 6 h and reaches a maximum after 48 h. This result was obtained on cells originating from different umbilical cords (n = 4). Moreover, the experiment was made in two ways, either after the addition of PMA at the zero time (e.g. 24 h after serum depletion) and collecting mRNAs after the indicated time of incubation or by collecting all mRNAs at the same end time (e.g. 48 h after depletion) and adding PMA at various times before collection. These two methods gave identical results, indicating that the cells remain in a homogeneous state during the whole experiment and were similarly responsive to PMA regardless of the serum depletion duration. Moreover, a control without PMA treatment was done for each time of incubation or by collecting all mRNAs at the same end time (e.g. 48 h after depletion) and adding PMA at various times before collection. These two methods gave identical results, indicating that the cells remain in a homogeneous state during the whole experiment and were similarly responsive to PMA regardless of the serum depletion duration.

We investigated the dose-response increase of ACE mRNA in response to PMA. The cells were incubated 24 h with various concentrations of PMA, and mRNAs were extracted before RPA analysis. As shown in Fig. 3, there is a marked increase in ACE mRNA from zero to 100 ng/ml PMA and a significant decrease of the PMA effect with 500 ng/ml. The concentration response for ACE mRNA level induction by PMA showed an EC50 of approximately 20 ng/ml. To determine if there was an increase in the transcription rate of ACE mRNA upon PMA treatment, run-on experiments were performed on isolated HUVEC nuclei. The results (Fig. 4) show that ACE gene transcription is undetectable without PMA stimulation, but a 6-fold increase in ACE mRNA transcription compared with control genes (β-actin or GAPDH) is observed after stimulation.

To confirm that the PMA-dependent stimulation of ACE involved activation of the PKC pathway, a PKC inhibitor (GF 109203X) was added to cultured HUVEC, either untreated or before PMA treatment. In PKC inhibitor-treated cells, we observed a dose-dependent inhibition of ACE mRNA level increase in the presence of PMA after 24 h (Fig. 5). The ACE/GAPDH ratio was significantly higher without PKC inhibitor than with 1 μM (10.2 ± 1.5 versus 3.2 ± 0.8, p < 0.01). When 5 μM inhibitor was added, the effect of PMA was nearly abolished. There was no significant difference in the ACE/GAPDH ratio between the control cells and the cells in the presence of 5 μM inhibitor.

Semiquantitative RT-PCR analyses of Egr-1 mRNA of PMA-treated cells (100 ng/ml) for different periods of time (30 min-20 h) were performed (Fig. 6). PMA induced Egr-1 transcript levels in HUVEC within 30 min of exposure, and Egr-1 mRNA levels remained elevated for 20 h with maximum levels (6-fold increase) reached between 1 and 2 h of PMA treatment. To test whether the PMA-dependent Egr-1 mRNA increase involved activation of PKC pathway, GF 109203X at a concentration able to inhibit ACE transcription by PMA (5 μM) was added to
cultured HUVEC under PMA treatment (100 ng/ml) (Fig. 6). In PKC inhibitor-treated cells, the effect of PMA was nearly abolished during the first 30 min of exposure, but the inhibition was not complete after longer exposure.

**DISCUSSION**

Treatment of HUVEC with PMA was responsible for a large increase in ACE secreted in the culture medium, in cellular ACE mRNA content, and in ACE transcription. These effects were mediated by PKC activation as shown by PKC inhibition experiments. The increase in ACE activity in the culture medium, observed after 24 h of PMA treatment, is in part the result of the proteolytic release of membrane-anchored ACE as shown previously after PMA stimulation of mouse epithelial cells or Chinese hamster ovary cells stably transfected with mouse or human testicular ACE cDNA, respectively (34, 35). This solubilization process, or membrane shedding, has been described for various growth factors, cytokines, or receptors such as protransforming growth factor-α, colony-stimulating factor-1, c-kit oncogene ligand, tumor necrosis factor-α receptors, L-selectin, or β-amyloid protein (36–42). This increase in secretion has been shown to be linked to PKC-dependent activation of a specific proteolytic mechanism in many cases (37, 38, 43). In the case of PMA-induced ACE secretion, it is a very rapid process that does not seem to be dependent on ACE gene transcription because it occurs when ACE is expressed under the control of a heterologous promoter (34, 35).

The important new finding reported in this study consists of the PMA-induced increase in endothelial cell ACE gene expression, which is associated with an increase in secretion but represents an independent process. The observed mRNA increase is nearly not yet detectable after 6 h of PMA treatment. This delay in the mRNA increase suggests that intermediate steps for mRNA induction are required because a post-translational mechanism, such as phosphorylation of preexisting transcription factors, would be expected to induce faster mRNA increase. Thus, a de novo expression of transcription factors is likely involved in ACE gene mRNA increase. The PMA induction of ACE mRNA lasted for 50 h, after which the effect was difficult to interpret because several factors were probably involved, such as PKC desensitization or cell injury by prolonged serum deprivation, as suggested by the increasing number of cells detached from the culture dishes after prolonged treatment periods.

Run-on experiments, performed on isolated HUVEC nuclei, show clearly that PMA induction of ACE mRNA acts at the transcriptional level. A well characterized signaling pathway of PKC is the activation of the AP1 transcription factor family, which interacts with the 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive-element (44), for which a consensus sequence is present in the 5′-flanking region of the ACE gene. Neverthe-
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In this study, a clear answer concerning the effect of PMA on ACE mRNA stability using actinomycin D could not be obtained. This was because of the low level of ACE mRNA expression in HUVECs in unstimulated conditions which did not allow the ACE mRNA to be quantified accurately after few hours of actinomycin D treatment. In addition, an unexplained increase in ACE mRNA was observed consistently after the 1st h of actinomycin D treatment when cells were stimulated by PMA, hampering the measurement of initial cellular ACE mRNA content. Despite these technical problems, no important differences between the ACE mRNA half-life of PMA-treated or control cells were detected (data not shown).

PKC activation represents a common pathway for several factors that are able to activate EC. These factors include hormones and growth factors such as vascular endothelial growth factor, basic fibroblast growth factor, and platelet-activating factor but also stressing conditions such as hypoxia or shear stress (26, 27, 58, 59). In response to this activation, EC synthesize several factors, such as intercellular adhesion molecule-1 (60), vascular cell adhesion molecule-1 (61), prostanand I2 (62), platelet-activating factor (63), platelet-derived growth factor-A and -B (64, 65), tissue factor (66), endothelin-1 (59), and cytoskeleton changes occur together with permeability changes (67, 68). This endothelial response to PKC thus represents a major step in the process of vascular diseases and may explain the ACE induction in conditions such as hypertension and diabetes.

Indeed, in the two-kidney-1clip hypertensive rat model of hypertension, ACE was shown to be increased in the vessel wall (69). The mechanical strain on the vessels walls, which is increased during hypertension, both on EC and vascular smooth muscle cells, is known to activate PKC in these two types of cells (70, 71) and might explain the ACE increase.

High glucose concentration is another condition that was shown to activate PKC in EC (25, 72). An increase in tissue and plasma ACE was observed during diabetes but was not explained clearly (73–76). According to our results one can hypothesize that ACE transcription might be induced by high glucose through PKC activation in EC. This mechanism could provide a biochemical basis for the beneficial effect of ACE inhibitor in preventing vascular complications of diabetes (77). Thus, ACE gene induction of expression by PKC activation might represent an important mechanism linking various diseases to ACE and the renin angiotensin system activation in vessels.

In summary, our results demonstrate a new transcription induction pathway of the ACE gene and its potential pathological implications. Additional experiments will allow the identification of PKC isoforms and the transcription factors involved in the ACE expression activation.

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**PKC Induction of ACE Gene Expression**

![Graph showing Egr-1 mRNA increase upon PMA treatment](image)

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