Phorbol Ester Induction of Angiotensin-Converting Enzyme Transcription Is Mediated by Egr-1 and AP-1 in Human Endothelial Cells via ERK1/2 Pathway

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Abstract—Angiotensin-converting enzyme (ACE) is an enzyme that plays a major role in vasoactive peptide metabolism, and it has been implicated in various cardiovascular diseases. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, has been shown to increase ACE mRNA at the transcriptional level in human umbilical vein endothelial cells. We have investigated the transcriptional mechanism involved in protein kinase C induction of the ACE gene. Deletion and transfection analyses have revealed that two regions are required for PMA-inducible gene expression. The first is a G+C-rich region located in the proximal ACE promoter bearing overlapping consensus recognition sequences for stimulatory protein-1 and early growth response (Egr)-1. Electrophoretic mobility shift assay and supershift experiments have shown that Egr-1 is present in the specific nucleoprotein complex induced by PMA in human umbilical vein endothelial cells. The second region is located in the distal ACE promoter. DNase I footprinting analysis restricted this region to a 21-bp element containing a cAMP-responsive element/12-O-tetradecanoylphorbol 13-acetate-responsive element sequence. Electrophoretic mobility shift assays and supershift analyses have revealed that activating protein (AP)-1 is the transcription factor binding the cAMP-responsive element/12-O-tetradecanoylphorbol 13-acetate-responsive element located in the ACE promoter after PMA stimulation. Mutations of either Egr-1 or AP-1 binding sites partially abrogate ACE expression induced by PMA, whereas mutation of both sites totally abrogates PMA-induced ACE expression. Treatment of cells with PD98059, a mitogen-activated protein kinase kinase-1–specific inhibitor, partially abrogate ACE expression induced by PMA, whereas mutation of both sites totally abrogates PMA-induced ACE expression. Our results demonstrate that the two transcription factors, Egr-1 and AP-1, are involved in the PMA-induced ACE transcriptional activation in human endothelial cells via the activation of the extracellular signal–regulated kinase 1/2 signaling pathway. (Circ Res. 2002;91:1547–1554.)

Key Words: mitogen-activated protein kinase ■ angiotensin-converting enzyme ■ transcriptional regulation ■ site-directed mutagenesis

Angiotensin I–converting enzyme (ACE; DCP1, EC 3.4.15.1) is a dipeptidyl carboxypeptidase that plays an important role in the renin-angiotensin system and kallikrein-kinin system. It is a zinc metallopeptidase that generates the vasoconstrictor peptide and growth factor angiotensin II from angiotensin I, inactivates the vasodilator peptide bradykinin, and is able to cleave several other peptides. Two isoenzymes of ACE are found in mammalian tissues, transcribed from a single gene by two alternate promoters. Somatic ACE (170 kDa) is synthesized by vascular endothelial cells and several epithelial cells, whereas testis ACE (110 kDa) is expressed exclusively by male germinal cells.

Modulation of ACE expression has been observed in response to various stimuli. ACE expression is induced by steroid hormones in macrophages and bovine pulmonary artery endothelial cells or by vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (HUVECs). ACE induction is also observed in several pathophysiological processes in different cell types. ACE expression is increased in early cardiac hypertrophy in rats. ACE has also shown to be induced in the aortic wall in different models of rat hypertension, such as the 2-kidney 1-clip hypertensive rat model, or in hypertension induced by chronic administration of the NO synthesis inhibitor N-nitro-L-arginine methyl ester.

However, only a few studies elucidating the molecular basis of ACE regulation have been performed. In a previous study, we have shown that phorbol 12-myristate 13-acetate (PMA)-activated protein kinase C (PKC) strongly increases ACE activity and ACE mRNA levels. Run-on experiments have demonstrated that PMA induction of ACE mRNA acts at the transcriptional level. Because PKC activation represents a common pathway for several factors able to activate endothelial cells and because some of them are known to induce ACE expression, elucidation of the transcriptional
mechanism involved in PKC induction of the ACE gene is of particular interest.

In the present study, we characterized the specific nuclear transcription factors that interact with functional cis elements in the ACE promoter in HUVECs stimulated with PMA. Our results demonstrate that an additive effect of two transcription factors, early growth response (Egr)-1 and activating protein (AP)-1, is involved in the PMA-induced ACE transcriptional activation via the Ras–mitogen-activated protein kinase kinase (MEK)–extracellular signal–regulated kinase (ERK) signal transduction pathway.

Materials and Methods

Cell Culture
HUVECs were isolated as described by Jaffe et al. Cells were grown in MCDB 131 medium (Sigma Chemical Co) supplemented with 20% heat-inactivated FCS, 2 mM/L i-glutamine, 100 U/mL penicillin-streptomycin, 10 ng/mL human epidermal growth factor (hEGF), and 1 μg/mL hydrocortisone. Cells were used between passages 2 and 3. Cells were maintained at 37°C and 5% CO, in a humidified incubator.

DNA Constructs
The ACE promoter–luciferase reporter genes were constructed by cloning a human genomic DNA fragment corresponding to −4335 to −1, which is relative to the transcriptional start site (GenBank accession No. AF229986), into the firefly luciferase reporter gene vector pGL3-basic (Promega). This construct was designated pACE-4335 and was digested by convenient restriction endonucleases for construction of deletion mutants as previously described by Testut et al. Several mutations were introduced in the pACE-1378 construct containing a fragment from −1378 to +1 of the ACE promoter was obtained by partially digesting pACE-2033 by exonuclease III/S1 nuclease using a commercial kit (Erase-a-Base system, Promega).

Expression Vectors
pSCTRk24, the expression vector for Egr-1, was a gift from Dr P. Charnay (École Normale Supérieure, Paris, France). Expression vectors for c-fos and c-jun were kindly provided by Dr M. Yaniv (Institut Pasteur, Paris, France).

Transient Transfection Assays
Cell transfection of plasmid DNA was carried out using polyethyleneimine suspension in a commercially available solution (EXGEN 500, Euromedex). HUVECs (250×10^3/well) were plated on a 6-well plate, incubated for 24 hours, and then treated with a buffer (150 mM/L NaCl) containing 0.37 pmol of the relevant reporter gene vector, 0.37 pmol of pRenilla luciferase gene vector (Promega) as the transfection-standardizing control, and 20 μL of EXGEN 500 in a final volume of 100 μL. Cells were incubated overnight with the transfection mix, deplated of serum (0.4%) and of hydrocortisone/hEGF for 16 hours before PMA activation. The nuclear extracts used to study Egr-1 binding and AP-1 binding were prepared as previously described by Khachigian et al and by Tamaru et al, respectively.

Electrophoretic Mobility Shift Assay
Nuclear extracts (5 μg) were incubated with ~0.15 pmol of [γ-32P]ATP end-labeled double-stranded oligonucleotides in the binding buffer containing 50 mM/L NaCl, 5 mM/L MgCl2, 5% glycerol, 2.5 mM/L HEPES, 1 μg poly(dl-dC), 20 μg BSA, 0.01% NP-40, 1 mM/L ZnCl2, 0.5 mM/L phenylmethylsulfonyl fluoride, and 1 mM/L diethiothreitol. Complexes were resolved by electrophoresis on prerun acrylamide/bisacrylamide (29:1) native gels.

DNase I Footprinting Analysis
A probe corresponding to a fragment of the ACE gene promoter region (−1435 to −1176) was obtained by polymerase chain reaction (PCR) amplification using oligonucleotides Sc1018I (sense, 5'-GACCCCGACTCTCTCTCCTC-3') and Br12a (antisense, 5'-TGAAGGGGGATGGTTGTCA-3'). For radioactive labeling, oligonucleotide Br12a (1.5 pmol) was end-labeled with [γ-32P]ATP by T4 DNA polynucleotide kinase (Life Technologies) before a 20-cycle PCR amplification using 10 pmol of the second primer, Sc1018I. DNase I footprinting analysis was performed by incubating 5000 cpm of probe with 50 μg of nuclear extracts for 20 minutes at 20°C, as previously described by Laumonnier et al.

Computer and Statistical Analysis
ACE gene promoter regions were checked for transcription factor binding sites using Transfac database and MatInspector 2.0 software. All transfection data were analyzed by ANOVA.

Results

Identification of Regions in ACE Promoter Required for PMA-Induced Gene Expression in Endothelial Cells

In previous investigations, ACE mRNA levels have been shown to be increased by activation of the PKC signaling system with the phorbol ester PMA. To identify cis-acting elements responsible for PMA-induced transcription of the ACE gene, a series of 5′-deletions of a 4335-bp promoter fragment were subcloned into the pGL3-basic vector containing the coding sequence of the firefly luciferase gene. These constructs were transiently transfected in HUVECs, and the promoter activities were measured by luciferase assay. As shown in Figure 1, cells transfected with pACE-4335 induced luciferase activity by 3-fold after treatment with PMA (100 ng/mL). Progressive deletions from −4335 to −1378 of promoter sequence (relative to transcriptional start site) did not significantly affect PMA-induced luciferase activity. Further deletion between −1378 and −1214 resulted in a significant attenuation of the induction by PMA from 3.9 to 2, respectively. A 2-fold induction was maintained with the smallest construct pACE-
132 bearing the first 132 bp of the ACE promoter. These results suggest that two PMA-responsive regions exist in the ACE promoter region in HUVECs. The first is a G+C-rich region localized between −132 and 1, and the second region is situated between −1378 and −1214.

**Protein Binding to G+C-Rich ACE Gene Promoter Region in Endothelial Nuclear Extracts**

Computer analysis of the G+C-rich ACE gene promoter region (−132/+1) revealed the presence of two overlapping sites for Egr-1/Sp1 transcription factors at −59 bp and −53 bp (Figure 2A). The binding activity of these sites in endothelial nuclear extracts was examined by electrophoretic mobility shift assay (EMSA). EMSA was performed with the use of a 32P-labeled double-stranded oligonucleotide corresponding to the −75 to −46 region of the ACE promoter (Oligo Ra, Figure 2A). A single PMA-induced nucleoprotein complex was observed when 32P-labeled oligonucleotide Ra was incubated with nuclear extracts from HUVECs exposed to 100 ng/mL PMA (Figure 2B, lanes 3 through 7). This complex is specifically competed by the presence of 50- to 100-fold molar excess of the unlabeled probe Ra (Figure 2B, lanes 8 through 11).

**Figure 1.** Mapping of PMA-responsive sequences in the human ACE promoter. HUVECs were transfected with 5′-deletion mutants and pRenilla luciferase gene vector as an internal control of transfection efficiency. After transfection, the cells were incubated with 100 ng/mL PMA for 24 hours at 37°C, and luciferase activities were measured using the Dual-Luciferase reporter assay system. The fold induction is defined as the ratio between PMA-induced firefly luciferase activity and basal firefly luciferase activity (both normalized with renilla luciferase activity). Results are mean±SE values of 4 experiments. *P<0.05 relative to the pACE-1378 construct.

**Figure 2.** Interaction of the G+C-rich element in the proximal ACE promoter with endothelial nuclear proteins exposed to PMA. A, Oligonucleotides used in EMSA are shown. Oligo Ra bears the nucleotide sequence between base pairs −75 and −46 of the human ACE promoter; the two overlapping Egr-1/Sp1 putative binding sites are indicated in boldface. Oligo Ra-m contains a mutated sequence abolishing the Egr-1/Sp1 binding sites. B, Labeled Ra oligonucleotide was incubated with 5 μg nuclear extract from unstimulated HUVECs or cells exposed to 100 ng/mL PMA for 0, 1, 3, 6, 12, or 24 hours at 37°C. Competition assays were performed with the use of oligonucleotides Ra, Ra-m, and Sp1 constructs (cons) from a ×50 to ×100 molar excess. C, Supershift (SS) analysis was performed with the use of 1 μL of the Egr-1 or the Sp1 antibody.
lanes 8 and 9). The addition of a molar excess of mutant competitor (Oligo Ra-m, Figure 2A), in which Egr-1/Sp1 binding sites were disrupted, did not interfere with the formation of the PMA-inducible complex (Figure 2B, lanes 10 and 11). The addition of an unlabeled oligonucleotide containing the Sp1 consensus motif (Figure 2A) did not compete for the formation of this complex (Figure 2B, lanes 12 and 13).

To identify the nuclear proteins present in the PMA-specific complex, antibody supershift experiments were performed. The PMA-inducible complex was no more detectable when the nuclear extracts were incubated with anti-Egr-1 antibody (Figure 2B, lane 18). The addition of an anti-Sp1 antibody did not affect the formation of any complexes (Figure 2B, lane 15).

All these results indicate that Egr-1 binds the −75 to −46 sequence of the ACE promoter after PMA exposure.

**DNase I Footprinting Analysis of ACE Gene Promoter Region (−1378/−1214)**

Computer analysis revealed the presence of a large number of putative binding sites for known transcription factors within the (−1378/−1214) region of the ACE promoter. DNase I footprint experiments were performed to determine which sequences in this region are able to bind nuclear proteins. An end-labeled PCR product corresponding to the (−1378/−1254) region of the ACE promoter was used as a probe in footprint experiments with HUVEC nuclear extracts stimulated or not with PMA. As shown in Figure 3A, both nuclear extracts from PMA-treated HUVECs and unstimulated cells protected only one element from DNase I degradation. This element spans ∼21 bp between −1322 and −1302 and includes a nonconsensus CRE/TRE between −1308 and −1315 (TGACGTGT).

**Protein Binding to the ACE Gene Promoter Region (−1322/−1302) in Endothelial Nuclear Extracts**

To determine whether the CRE/TRE is able to bind nuclear proteins from PMA-treated HUVECs, we performed a series of competition band-shift and supershift experiments.

The 32P-labeled double-stranded oligonucleotide (Oligo Rb, Figure 3B) bearing the 21-bp element protected in footprint analysis binding activity of the CRE/TRE was analyzed. A single PMA-induced nuclease protein complex was observed when 32P-labeled oligonucleotide Rb was incubated with nuclear extracts from HUVECs exposed to 100 ng/mL PMA (Figure 3B, lanes 4 through 7). The addition of a molar excess of the unlabeled probe Rb led to the disappearance of this complex, whereas a molar excess of a mutant competitor with a disrupted CRE/TRE (Oligo Rb-m) did not compete for the formation of this complex (Figure 3B, lanes 8 through 11). Furthermore, the PMA-inducible complex did not appear when a 32P-labeled oligonucleotide Rb-m was incubated with nuclear extracts from HUVECs exposed to PMA (Figure 3B, lanes 12 through 14).

To identify the transcription factors binding the CRE/TRE sequence in nuclear extracts of PMA-treated HUVECs, supershift experiments were performed using a CREB/ATF antibody, reactive with activating transcription factor-1 (ATF-1), CREB-1, and CRE-modulator protein-1 (CREM-1), and a c-fos and a c-jun antibody. Only the addition of a c-jun antibody completely blocked the formation of the PMA-inducible complex (Figure 3B, lanes 15 through 20). These results suggest that an AP-1 complex containing a c-jun homodimer is able to bind the CRE/TRE sequence of the ACE promoter in response to PMA.

**Functional Analysis of Egr-1 and AP-1 Binding Sites of the ACE Gene Promoter**

To test the functionality of Egr-1 and AP-1 binding sites, eucaryotic expression vectors encoding Egr-1, c-jun, and c-fos or an empty control vector (pCDNA3.1) were cotransfected with pACE-1378 in HUVECs, and the promoter activity was assessed. Coexpression of the expression vector encoding for Egr-1 (Figure 4A) or c-jun alone (Figure 4B) dose-dependently activated the pACE-1378 transcriptional activity, whereas coexpression of c-jun and c-fos (Figure 4B) had no effect on the transcriptional activation of pACE-1378. These results suggest that both Egr-1 and c-jun functionally interact with the ACE promoter to induce ACE expression but that c-fos is not involved.

We prepared a set of site-directed mutants with sequence variations in either or both the Egr-1 and the AP-1 binding sites. The mutations were introduced in the context of the p1378 construct that had a full functional activity in response to PMA by use of a PCR-based mutagenesis with mutant oligonucleotides. These constructs were transfected in HUVECs, and PMA-induced promoter activity was determined (Figure 5). PMA-stimulated luciferase activity of the pACE-1378m1 construct, in which the Egr-1 binding site in position −59 was disrupted, was 1.85-fold. Interestingly, disruption of the second Egr-1 binding site in position −53 (pACE-1378 m2) had no effect on ACE promoter activity in response to PMA. Indeed, transfection with pACE-1378 m2 resulted in a 2.63-fold activation of luciferase activity in response to PMA, which was not significantly different from the 3-fold increase observed with the wild-type construct. HUVECs transfected with the pACE-1378 m3 construct, with a mutated CRE/TRE sequence, expressed a luciferase activity that was 1.8 higher when exposed to PMA. To determine whether only the Egr1(−59) binding site and the CRE/TRE participate in the PMA-inducible expression of the ACE gene, we made a reporter construct in which both sites were disrupted, pACE-1378 m4. These mutations totally abolished the PMA-promoted induction.

These findings demonstrate the requirement of both intact Egr-1 (in position −59) and CRE/TRE (in position −1308 to −1315) binding sites in the ACE promoter sequence for PMA-inducible expression.

**ERK1/2 Pathway Is Involved in PMA-Induced ACE Expression**

The contribution of ERK1/2 activation to the PMA-mediated transcriptional activity of the ACE gene promoter was assessed in cells transfected with the pACE-1378 construct and pretreated for 30 minutes with PD98059, a MEK-1–specific inhibitor (5 μmol/L in 0.1% dimethyl sulfoxide [DMSO]), or...
with 0.1% DMSO alone before being subjected or not to PMA treatment (100 ng/mL) for 24 hours. The data in Figure 6A show that under these experimental conditions, PMA increased luciferase activity by 3-fold. PD98059 totally inhibited this effect, suggesting that PMA signal transduction pathway in HUVECs requires activated MEK-1. The inhibitory effect of PD98059 on PMA-induced luciferase activity was observed when cells were transfected with either pACE-1378m1, whose Egr-1 (−59) binding site is disrupted, and pACE-1378m3, whose AP-1 binding site is disrupted. These results suggest that the ERK1/2 pathway is involved in the activation of both transcription factors (Egr-1 and AP-1), thus leading to ACE transcriptional induction in response to PMA in HUVECs.

We determined the effects of PD98059 on PMA-induced interaction between Egr-1 and AP-1 proteins with the ACE promoter. Nuclear extracts from HUVECs treated with 0.1% DMSO, PD98059 (5 μmol/L in 0.1% DMSO), PMA (100 ng/mL in 0.1% DMSO), or both PD98059 and PMA were incubated with the 32P-labeled oligonucleotide Ra (Figure 6B, Figure 3. In vitro endothelial nuclear protein interactions with the Rb region of the human ACE promoter. A, DNase I footprint analysis of the −1435 to −1176 region of the ACE promoter. A DNA fragment spanning 259 bp was PCR-amplified with a labeled primer and submitted to increasing amounts of DNase I endonuclease in the absence (lanes 2 through 4) or in the presence of nuclear extracts from HUVECs submitted to PMA treatment (100 ng/mL) for 0, 1, 3, 6, 12, or 24 hours (lanes 5 through 17). The A+G sequence marker (lane 1) was obtained by Maxam-Gilbert sequencing of the end-labeled fragment. The protected element is boxed, and the corresponding nucleotide sequence is indicated. B, Interaction of the CRE/TRE in the ACE promoter with endothelial nuclear proteins exposed to PMA. Oligo Rb bears the nucleotide sequence between base pairs −1325 and −1296 in the human ACE promoter; the CRE/TRE is indicated in boldface. Oligo Rb-m is mutated on the CRE/TRE sequence. Labeled Rb oligonucleotide or labeled Rb-m was incubated with 5 μg nuclear extracts from unstimulated HUVECs or cells exposed to 100 ng/mL PMA for 0, 1, 3, 6, 12, or 24 hours at 37°C. Competition assays were performed with the use of oligonucleotides Rb or Rb-m from a ×10 to ×25 molar excess. SS analysis was performed using 1 μL of the antibody (Ab) indicated.
lanes 1 through 8) or with the 32P-labeled oligonucleotide Rb treatment induced the formation of a specific nucleoprotein complex that was no more visible in the presence of nuclear

Figure 4. Effect of the coexpression of Egr-1, c-fos, and c-jun on the transcriptional activity of the pACE-1378 construct. HUVECs were cotransfected with an expression vector for Egr-1 (A) or an expression vector for c-jun alone, both c-jun and c-fos expression vectors, or an empty vector (pcDNA3.1) (B) and the pACE-1378 construct and pRenilla luciferase gene. After transfection, the cells were incubated for 24 hours at 37°C, and luciferase activities were measured using the Dual-Luciferase reporter assay system. Relative luciferase activity is defined as the ratio between firefly luciferase activity and renilla luciferase activity. Results are mean±SE values of 3 experiments. *P<0.05 relative to the empty vector.

Figure 5. Transcriptional activities of the site-directed mutants of either or both Egr-1 and AP-1 binding sites in HUVECs. A, Sequences of the site-directed mutants are shown. Mutations of the Egr-1(-53), Egr-1(-59), or AP-1 binding sites were introduced in the pACE-1378 construct. Binding sites for Egr-1(-53), Egr-1(-59), or AP-1 are indicated in boldface; mutations introduced are underlined. B, HUVECs were transfected with mutant constructs and pRenilla luciferase gene vector. After transfection, the cells were incubated with 100 ng/mL PMA for 24 hours at 37°C, and luciferase activities were measured using the Dual-Luciferase reporter assay system. Fold induction is defined as the ratio between PMA-induced firefly luciferase activity and basal firefly luciferase activity, with both normalized with renilla luciferase activity. Results are mean±SE values of 4 experiments. *P<0.05 relative to the pACE-1378 construct.
extracts treated by both PMA and PD98059. Supershift experiments confirmed that the nucleoprotein complex formed in the presence of the $^{32}$P-labeled oligonucleotide Ra contains Egr-1, whereas the nucleoprotein complex formed in the presence of the $^{32}$P-labeled oligonucleotide Rb contains c-jun. These results indicate that ERK1/2 is directly involved in the binding of the Egr-1 and AP-1 transcription factors to the ACE promoter after PMA stimulation.

**Discussion**

Our previous study showed that PMA treatment was responsible for increasing ACE transcription in HUVECs. In the present study, we investigated the molecular mechanisms of the PMA-regulated ACE expression in endothelial cells.

A 4335-bp fragment of the ACE promoter was analyzed for the presence of PMA-responsive elements. $5'$-Deletion analysis of the ACE promoter indicates that two distinct regions are required for PMA-induced ACE expression. The first is a G+C-rich region located in the proximal promoter (−132/+1). This region includes two putative binding sites for the Egr-1 transcription factor in positions −53 and −59, overlapping with two Sp1 potential binding sites. EMSA using PMA-treated endothelial cell nuclear extracts demonstrated that the formation of a nucleoprotein complex was induced. Supershift analysis revealed that Egr-1 is the nuclear factor that binds specifically to this region of the ACE promoter. The zinc-finger transcription factor Egr-1 is an “immediate-early response protein” that is rapidly and transiently induced by a large number of growth factors, cytokines, and injurious stimuli. A model for the cascade of molecular events underlying the inducible expression of Egr-1–dependent genes in vascular endothelial cells has been proposed: multiple extracellular stimuli activate phosphorylation-dependent signaling pathways, which stimulate Egr-1 expression and activation. In the nucleus, Egr-1 binds to an element overlapping the Sp1 site, displaces the prebound Sp1, occupies the GC box, and stimulates transcription of the target gene. This mechanism has been proposed for PMA-stimulated expression of platelet-derived growth factor-B or tissue factor gene, but other genes, such as the basic transcription regulatory element binding protein 2, have been shown to be regulated by Egr-1 in response to PMA stimu-
lation in vascular smooth muscle cells, without Sp1 displacing.19 In the case of the ACE promoter, Egr-1 seems not to displace prebound Sp1 because appearance of the Egr-1 complex is not accompanied by displacement of another complex and because none of the observed complexes are affected by the presence of a molar excess of a consensus sequence for the Sp1 binding site or an Sp1 antibody.

EMSA experiments showed that PMA-treated nuclear extracts bind the two overlapping Egr-1 binding sites found at positions −253 and −259. In contrast, only the −259 binding site is functional because the mutation of this site decreased by 40% the induction of ACE in response to PMA, whereas mutation of the putative binding site in position −53 did not affect this response.

Footprinting analysis of the region (−1378 to −1214) implicated in PMA regulation revealed a protected element between −1322 and −1302, including a nonconsensus CRE/TRE sequence. Consensus sequences of CRE (TGACGTCA) and TRE (TGAGTCA) are closely related, inasmuch as they differ by a single nucleotide. The sequence of the CRE/TRE-like element within the ACE gene promoter (TGACGTGT) differs by two nucleotides from the CRE consensus and by three from the TRE consensus sequence. The consensus CRE sequence is known to bind CREB, whereas the consensus TRE sequence preferentially binds AP-1. However, the similarity between the two regulatory elements may allow cross talk between components of these two major signal transduction pathways.20 EMSA revealed that PMA induces the formation of a nucleoprotein complex on the CRE/TRE sequence. This EMSA complex is not visible with nuclear extracts not treated with PMA, whereas the protected element detected by footprinting experiments was visible with nuclear extracts that were treated or not with PMA. One explanation could be that other proteins could bind this region of the ACE promoter independently of the presence of PMA; this is consistent with the fact that two nucleoprotein complexes are visible in EMSA with nuclear extracts not submitted to PMA treatment. The nature of the proteins involved in these complexes was not determined. To identify the nuclear factor involved in the formation of the PMA-induced complex, supershift analysis was performed. A CREB/ATF antibody that reacts with ATF-1, CREB-1, and CREM-1 had no effect on the mobility of the PMA-induced complex. A c-jun antibody is able to block the formation of this complex, whereas a c-fos antibody has no effect. This result indicates that an AP-1 complex transcription factor formed by a jun homodimer binds the ACE promoter on the CRE/TRE in position −1308 on PMA exposure. Treatment by phorbol ester is one of the extracellular stimuli known to activate the mitogen-activated protein kinase signaling pathway.21 Furthermore, AP-1 and Egr-1 are known to be activated by the ERK1/2 or the c-Jun NH2-terminal kinase (JNK) pathway in different cell types.22,23 We found that transfection of a dominant-negative form of JNK has no effect on the transcriptional activation of ACE in response to PMA (please see online expanded Materials and Methods section), whereas the MEK-1–specific inhibitor PD98059 totally abolishes the response of the ACE promoter to PMA in a reporter construct, suggesting that signal transduction via ERK1/2 is predominantly involved in the PMA activation of ACE.

Figure 7. Schematic drawing of the proposed mechanism for PMA-induced ACE transcription. PMA-activated PKC activates the ERK1/2 signaling pathway in a manner dependent or not dependent on Ras. ERK1/2 phosphorylates the ternary complex factor that binds the SRE Egr-1 gene, causing an induction of this gene expression. ERK1/2 is also able to activate c-jun, which forms the AP-1 complex composed by the homodimer jun/jun. AP-1 and Egr-1 transcription factors bind their target sequence in the promoter of the ACE gene, causing an upregulation of ACE expression. TCF indicates ternary complex factor.
transcription through both the AP-1 and Egr-1 response elements present in the 1378-bp promoter fragment. In HUVECs, PD98059 at the same concentration (5 μmol/L) does not completely inhibit the induction of ACE mRNA expression (please see online data supplement). The cascade of events leading to transcriptional ACE upregulation in response to phorbol ester stimulation is summarized in Figure 7.

The PKC signaling is a convergent path for various extracellular stimuli; thus, the new transcription induction pathway of the ACE gene described in the present study might represent a mechanism of ACE gene activation in various pathophysiological processes that involve PKC activation. A direct link between growth factor stimulation, PKC activation, and ACE upregulation has recently been established in endothelial cells. VEGF was shown to induce ACE mRNA levels in HUVECs cultured in medium in the presence or absence of VEGF (50 ng/mL). Thus, ACE upregulation induced by VEGF and mediated by PKC seems not to act through a transcriptional mechanism involving the first 1378 bp of the ACE promoter in HUVECs. Shear stress is another pathophysiological stimulus candidate for being involved in ACE upregulation through the molecular pathway described in the present study. Various arguments are in favor of this hypothesis. First, ACE expression is upregulated by shear stress in the vascular cells of rats. Second, PKC is one of the intracellular mediators of the shear stress signal transduction pathway. Furthermore, the molecular cascade of signalization that we described shares many similarities with transduction pathways known to mediate the transcriptional regulation of various genes in response to shear stress. Thus, the ERK1/2 pathway is activated by shear stress in vascular endothelial cells, leading to the activation of Egr-1 and AP-1 transcription factors. In summary, we demonstrated the functional role of two major elements for transcriptional regulation of ACE. These elements could participate in the modulation of ACE expression observed in major physiological and pathophysiological conditions.

Acknowledgments

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**Expression vectors** - The wild-type (JNK-wt) and dominant negative (JNK-DN) expression vectors for JNK was a gift from Dr B.Derijard (UMR, CNRS 6548, Nice, France)

**RT-PCR analysis** - Total RNA was extracted using Trizol reagent (Life Technologies) as described by the manufacturer instructions. 500 ng of RNA was used to reverse transcription using M-MLV reverse transcriptase (Life Technologies) for 50 min at 37°C. Following oligonucleotides were used to amplify the human ACE gene and the actin gene : 49 (sense), 5'-CACTCTACCTCAACCTGC-3' and 56 (antisense) 5'-AAGGGAAAGGGCACCACCAAG-3', Act5' (sense), 5'-ATTCCTATGTGGGCGACGAGG-Act3' (antisense) 5'-GCCTGGATAGCAACGTACATG-3'. Products of amplification (5µl) were resolved by electrophoresis on 5% acrylamide:bisacrylamide (29:1) native gels. Autoradiographic signals were quantitated by scanning with a laser densitometer (FUJI Phosphorimager).

**JNK pathway is not involved in PMA-induced ACE expression**

The contribution of JNK activation to the PMA mediated transcriptional activity of the ACE gene promoter was assessed by co-transfection of a wild-type, a dominant negative form of JNK or an empty vector and the pACE-1378 construct. The results indicate that PMA increased luciferase activity of the pACE-1378 construct is equivalent in the presence of the empty vector, the wild-type or the dominant negative form of JNK. Same results were observed when these expression vectors were co-transfected with either pACE-1378m1 whose Egr-1 (-59) binding site is disrupted, and pACE-1378m3 whose AP1 binding site is disrupted. These results suggest that the JNK pathway is not involved in the PMA-transcriptional activation of ACE.
**Effect of ERK1/2 inhibition on PMA-induced ACE expression**

Levels of ACE and actin mRNA from cells pretreated with PD 98059 (5 µM in 0.1% DMSO) or with 0.1 % DMSO alone for 30 min before PMA-treatment of 24 h were determined by RT-PCR analysis and quantified by densitometry. A non significative induction of 1.5 fold of ACE expression is observed in cells treated with PD98059 whereas in non treated cells PMA induced ACE expression by 4 fold. Thus, the induction of ACE mRNA level by 24h of PMA treatment (100 ng/ml) was notably reduced in the presence of 5 µM of PD98059 but not totally abolished.. Although this residual activation of ACE expression was abolished by increasing the concentration of PD98059 (from 5 to 50 µM, data not shown), we can not exclude that another pathway of ACE expression induction, distinct from Ras-MEK-ERK cascade, exists in response to PMA.

**Fig.A** HUVEC were co-transfected with pACE-1378, pACE-1378m1 or pACE-m3 constructs and with an empty vector (black bars), a wild-type expression vector of JNK (white bars) or a dominant negative expression vector of JNK (grey bars). Luciferase activity was measured using Dual-luciferase reporter assay system (Promega). The fold induction is defined as the ratio between PMA-induced firefly luciferase activity and basal firefly luciferase activity both normalized with renilla luciferase activity. Results are means ± S.E of three experiments.

**Fig.B** ACE and actin mRNA levels of HUVEC exposed to PMA (solid bars) or not (open bars) and submitted or not to PD98059 treatment, were determined by radioactive RT-PCR analysis and quantified by densitometry using a PhosphorImager (Fuji). Results are expressed as a ratio of densitometry values between ACE and actin, and are means ± S.E of four experiments*, p<0.05, control cells treated with 0.1% DMSO.