

Gene Expression Profiling of Inflamed Human Endothelial Cells and Influence of Activated Protein C

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Background—During systemic inflammation, activation of vascular endothelium by proinflammatory cytokines leads to hypotension, microvascular thrombosis, and organ damage. Recent data suggest a link between coagulation and inflammation through the activated protein C (APC) pathway. We studied gene expression profiles in human coronary artery endothelial cells (HCAECs) exposed to proinflammatory stimuli and the influence of APC on expression of candidate genes regulated by these stimuli.

Methods and Results—HCAECs were stimulated with interleukin-1 β , interferon- γ , and tumor necrosis factor- α . In gene expression profiling, 400 of 8400 genes were regulated >2-fold. Verification of selected candidate genes was achieved by measuring expression of mRNA species by real-time polymerase chain reaction, cytokine secretion by ELISA, and metabolites of tetrahydrobiopterin (BH4) biosynthesis by high-performance liquid chromatography. BH4 synthesis, interleukin-6, interleukin-8, monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) were downregulated by APC at the transcriptional and protein level. Endothelial nitric oxide synthase, endothelial adhesion molecule, and vascular cell adhesion molecule-1 were not affected by APC. Activities of transcription factors c-Fos, FosB, and c-Rel were inhibited by APC in inflamed HCAECs.

Conclusions—Our study revealed a novel antiinflammatory mechanism of APC-dependent gene regulation in HCAECs since c-Fos–dependent induction of MCP-1 and ICAM-1 was suppressed. APC downregulates expression and activity of genes related to inflammation, most pronounced under intermediate or mild inflammatory conditions. (*Circulation*. 2004;110:2903-2909.)

Key Words: endothelium ■ genes ■ inflammation ■ interleukins ■ cell adhesion molecules

The endothelial cells line the vessel wall and play critical roles in controlling the vascular tone and in immunovascular communication by interacting with circulating cells such as monocytes and platelets. Endothelial function is profoundly altered by systemic inflammation. Interleukin (IL)-1 β , tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) are produced in response to inflammatory conditions, trigger the inflammatory cascade, and play a major role in the activation of endothelium.¹

This cellular activation results in dysregulated endothelial function, leading to microvascular thrombosis, organ damage, multiple organ dysfunction, and often death.² Expression of adhesion molecules, proinflammatory cytokines, signaling pathway molecules, oxidation products, and possibly tissue factor (TF) and induction of apoptosis, nitric oxide production, and activation of transcription factors are stimulated in dysfunctional endothelial cells.³ TF promotes the inflamma-

tory pathway by promoting thrombin generation, which activates endothelial cells and platelets.⁴

Most cell-based studies of endothelial function use human umbilical vein endothelial cells (HUVECs). These cells are derived from a vascular bed that does not exist in adults. The process of endothelial dysfunction in inflammation takes place in the microvascular bed; therefore, a HUVEC model may not represent a valid model for endothelial function of inflammation in adults. Furthermore, endothelial cell populations differ depending on their anatomic location.⁵

In patients with systemic inflammation and sepsis, disseminated intravascular coagulation (DIC) is observed because of aberrant TF expression on monocytes and an altered proadhesive endothelial environment. Clinical trials targeting the inflammatory cascade with molecules such as IL-1, TNF- α , or lipopolysaccharide were unsuccessful in treatment of sepsis. In systemic inflammation, the proinflammatory stim-

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TABLE 1. Cytokine Combinations Used in This Study

Stimulus	IL-1 β , U/mL	TNF- α , U/mL	IFN- γ , U/mL
S1	20	100	100
S2	10	50	50
S3	10	20	20
S4	5	5	10
S5	2.5	5	5

uli are present not as single factors but combined in different concentrations. Therefore, when influencing a single proinflammatory trigger, its function will be replaced because of redundancy in this system. Trials replacing several different coagulation proteins improved DIC but not the systemic inflammatory response and death rate.⁶ A recent clinical trial using recombinant human activated protein C (rhAPC) in patients with severe sepsis not only improved DIC but also prevented death,⁷ indicating that rhAPC not only acts as an anticoagulant protein but also may have antiinflammatory properties. Protein C is a circulating zymogen that is activated to activated protein C (APC) on binding to the endothelial thrombin/thrombomodulin complex. APC is a serine protease that inactivates the clotting factors FVIIIa and FVa. Studies using a HUVEC-based model suggested that rhAPC has antiinflammatory effects.⁸ However, targets of rhAPC induced in inflammation have not yet been studied in cell-based experiments with primary human vascular endothelial cells.

In our present study we use a primary microvascular endothelial cell system, human coronary artery endothelial cells (HCAECs). To identify candidate genes to be investigated with rhAPC, we first performed a gene expression profiling experiment with HCAEC stimulated with the most potent proinflammatory cytokine mixture, IL-1 β , IFN- γ , and TNF- α .⁹ The effects of rhAPC on target gene expression and function were then studied in subsequent experiments. In these experiments, the effect of rhAPC was investigated in HCAECs activated with cytokine mixtures representing severe to mild inflammatory conditions.

Methods

Cell Culture

HCAECs were cultured in EGM-2-MVBulletkit (Clonetics, Cambrex) in humidified air with 5% CO₂ at 37°C. Cells from passages 3 to 6 were seeded in T-75 flasks or 6-well plates (Falcon, Becton Dickinson). At 90% confluence, cells were incubated with fresh medium containing rhIL-1 β , rhTNF- α , and rhIFN- γ (Pepro Tech) for 6 hours at concentrations indicated in Table 1. RhAPC (Xigris, Lilly) was added as indicated in Results and figure legends. This rhAPC preparation is registered by the Food and Drug Administration for use in patients, and the Food and Drug Administration reports document no evidence of lipopolysaccharide. In medium not exposed to cells and in medium exposed for 24 hours to unstimulated and stimulated cells, APC activity was not detected by chromogenic assay (described below). To maintain the rhAPC concentration in culture media at 3.6 to 7 μ g/mL, addition of 5 μ g/mL rhAPC to the cultures every 12 hours was necessary. In our system, thrombin and other protease activities were not present in all experiments involving rhAPC.

Gene Expression Profiling

HCAECs (6×10^7 cells) were treated for 6 hours with cytokine mixture S1, as indicated in Table 1. Cells were detached with the

ReagentPack (Clonetics, Cambrex), washed with PBS (Gibco), and cryopreserved at -70°C. Gene expression profiling comparing stimulated with unstimulated HCAECs was performed with the use of BD Atlas Plastic Human 8K Microarray Customer Service (Clontech).

RNA was purified with AtlasPure Total RNA Labeling System (Clontech). To assess reproducibility of the arrays, duplicate samples were labeled and hybridized twice for each treatment. Data analysis was performed with the use of Atlas Image 2.0 (Clontech). To define the hybridization efficiency, the sum of all signals in all spots was measured and divided by the number of spots. Each gene was represented by 2 spots on the array. The sum of the hybridization intensity of these 2 spots was taken and divided by the hybridization efficiency. If a spot had intensity <20, it was considered background. A ≥ 2 -fold change in hybridization intensity between control and stimulated HCAECs was considered significant. Functional gene clustering was performed with the use of software provided by Clontech at www.atlas.clontech.com.

Quantitative Real-Time Polymerase Chain Reaction

For the genes of interest, confirmation of regulation was achieved by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) as described previously.¹⁰ Specific primers used are listed in Table I (online only). These primers were designed with respect to the chemical and experimental conditions of the LightCycler technology used to prevent primer-dimer formation. For this, the specialized primer design software supplied by the manufacturer was used (Roche Probe Design 1.0). Melting curve analysis performed simultaneously in each PCR experiment detects primer-dimer formation if present. Measurement of primer-dimers can be excluded by melting away primer-dimers before acquisition of fluorescence at the end of each cycle.

Cycling conditions were an initial denaturation (10 minutes, 95°C), followed by 45 cycles of denaturation (15 seconds, 95°C), annealing (10 seconds, 55°C to 67°C, as indicated in Table I [online only]), and extension (12 seconds, 72°C), with acquisition of fluorescence at the end of each extension. All products were sequenced to ascertain specific amplification (Microsynth Customer Service, Balgach).

mRNA expression was quantified with the use of external standards of the respective PCR products purified with Qiaquick PCR Purification Kit (Qiagen). PCR products were spectroscopically quantified, and 5-fold serial dilutions were used as standards. Real-time PCR data were analyzed with LightCycler analysis software, version 3.5. The results are normalized to the housekeeping gene GAPDH and expressed as fold induction in treated versus untreated cells. Details of the relative quantification with external standards have been described previously.^{11,12} Differences of ≥ 2 -fold in mRNA expression between stimulated and unstimulated samples are considered significant.

APC Activity Assay

Serine protease activity of rhAPC in cell culture supernatants was measured with the use of chromogenic substrate S-2366 (Chromogenix). RhAPC proteolytic activity was quantified after addition of 4 mmol/L CaCl₂, 0.2% BSA (Sigma), and 1 mmol/L S-2366 for 10 minutes at 37°C. A standard curve was generated by serial dilution of rhAPC in Tris-buffered saline, pH 8.3, containing 0.2% BSA. Samples were measured in duplicate by end-point assay after addition of 20% acetic acid (Fluka) and read at 405 nm in a UV-Vis Plate Spectrophotometer (Spectra Count, Packard).

Western Blot Analysis

HCAECs (2×10^6 cells) were washed with PBS and scraped from the flask with 500 μ L of boiling Laemmli Sample Buffer (Bio-Rad Laboratories) and 5% β -mercaptoethanol (Sigma). Suspensions of equal cell numbers were passed several times through a 26-gauge needle and centrifuged at 1000 rpm in an Eppendorf microfuge for 5 minutes. Thirty microliters of the supernatant was used in 7.5% SDS-PAGE (Bio-Rad Laboratoires AG). After blotting onto nitro-

cellulose (Bio-Rad Laboratoires AG), membranes were incubated with endothelial nitric oxide synthase (eNOS)/NOS type III (BD Transduction Laboratoires) monoclonal antibody. Blots were developed with the ECL System (Amersham Biosciences). Quantification of bands was achieved with the Chemidoc XRS imaging system with the use of QuantityOne analysis software version 4.4 (Bio-Rad Laboratoires).

Cytokine ELISAs

IL-6, monocyte chemoattractant protein-1 (MCP-1), and IL-8 were quantified in HCAEC tissue culture supernatants in duplicate at several dilutions according to the manufacturer's instructions (Quantikine, R&D System) with the use of an end-point method on a Spectra Count (Packard) ELISA reader. Detection limits of the assays were 5.0 pg/mL for MCP-1, 3.5 pg/mL for IL-8, and 0.7 pg/mL for IL-6.

Nuclear Extract

HCAECs (6×10^7 cells) were treated with cytokine mixture S4 (Table 1) with and without 5 $\mu\text{g/mL}$ rhAPC for 2 hours. Nuclear extract was prepared with the use of Transfactor Extraction Kit (Clontech BD Biosciences). Briefly, cells were treated in a hypotonic buffer and homogenized. The cytosolic fraction was collected after pelleting of membrane debris. The nuclear fraction was obtained by shrinking the nuclei, followed by further homogenization and pelleting of the nuclear membrane debris.

Transcription Factor Activity Assay

Detection of transcription factor activity was performed with the BD Mercury TransFactor Profiling Kits, Inflammation 1 and 2 (Clontech BD Biosciences), which use an ELISA-based technique as described.¹³

Measurement of Pteridines

Pteridines were quantified by high-performance liquid chromatography after acidic oxidation, as described previously.¹⁴

Statistical Analysis

Results are expressed as mean \pm SEM. Data were analyzed with the use of Graphpad Prism version 4.0 statistical software. An unpaired 2-tailed Student *t* test or, for comparison of multiple treatment groups, a 1-way ANOVA with Dunnett posttest was used. Differences were considered statistically significant at $P < 0.05$.

Results

Gene Expression Profile

We first studied the gene expression profile of HCAECs exposed for 6 hours to S1 (Table 1), representing the most potent proinflammatory cytokine mixture,⁹ by microarray analysis. Of the 8400 genes analyzed, we found that 4.7% were regulated on stimulation. We found that 85% of the regulated genes were induced, and 15% were suppressed. As shown in Table 2, the highest numbers of regulated genes were found in the functional clusters for adhesion, transcription factors, cell signaling, intracellular transducers, and metabolism, representing >30 genes changing per cluster on stimulation. A list of regulated genes clustered according to functional groups is presented in Table II (online only). In a candidate gene approach, we then investigated the effect of rhAPC on selected regulated genes such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule (ELAM), IL-6, IL-8, MCP-1, and nuclear factor κB (NF κB).

TABLE 2. Genes Regulated by Proinflammatory Cytokine Combination S1 (Table 1) in HCAECs

	Clustered Regulated Genes	
	Up	Down
Total genes analyzed: 8400	340	60
Surface, adhesion, receptors	36	12
Transcription factor	40	2
Apoptosis associated	6	3
Cell signaling	28	6
Intracellular transducer	18	14
Metabolism	50	10
Cell cycle	5	0
Immune system	5	0
Posttranscriptional modification	16	3
Transport, carrier proteins	24	5

Only genes regulated ≥ 2 -fold were included.

Effect of rhAPC on HCAEC Morphology

The morphology of HCAEC changed dramatically after stimulation for 48 hours with proinflammatory cytokines (S1 mixture; Table 1). The cells became elongated, and gaps appeared. The addition of rhAPC to the proinflammatory cytokine mixture improved the altered cell morphology but did not restore it to the normal cobblestone appearance of untreated cells. RhAPC (5 $\mu\text{g/mL}$) alone did not alter cell morphology of unstimulated cells (data not shown).

Effects of rhAPC on Gene Expression of Inflamed HCAECs

Effect of rhAPC on Tetrahydrobiopterin Synthesis

In a first series of experiments, we tested the effect of rhAPC on the tetrahydrobiopterin (BH4) synthesis enzymes GTP cyclohydrolase I (GTPCH) and 6-pyruvoyl tetrahydropterin synthase (PTPS), known to be upregulated in endothelial cells stimulated with IL-1 β , TNF- α , and IFN- γ .^{9,15} In a first experiment, HCAECs were exposed to the most potent proinflammatory mixture, S1 (Table 1), in the presence of different concentrations of rhAPC, as indicated in Figure 1A. A 30% decrease in GTPCH mRNA expression was achieved with the lowest rhAPC dose. Higher doses did not further reduce GTPCH mRNA expression in our system. Therefore, we chose a dose of 5 $\mu\text{g/mL}$ for all further experiments.

In a second experiment, the specificity of the observed effect was studied by using denatured rhAPC, which had no remaining proteolytic activity in a chromogenic assay (data not shown). HCAEC stimulated with cytokine mixture S2 (Table 1) showed a 180-fold induction of GTPCH mRNA expression, which was reduced by 50% after the addition of rhAPC (Figure 1B). Denatured rhAPC (95°C for 15 minutes) had no effect on GTPCH mRNA expression, indicating the requirement of an active serine protease for its activity.

In a third experiment, the effect of rhAPC on GTPCH mRNA expression in HCAECs treated with different cytokine mixtures S1 to S5 (Table 1) was investigated (Figure 1C). S1 represents the most severe stimulus, and S5 is a mild proinflammatory stimulus. GTPCH mRNA expression was

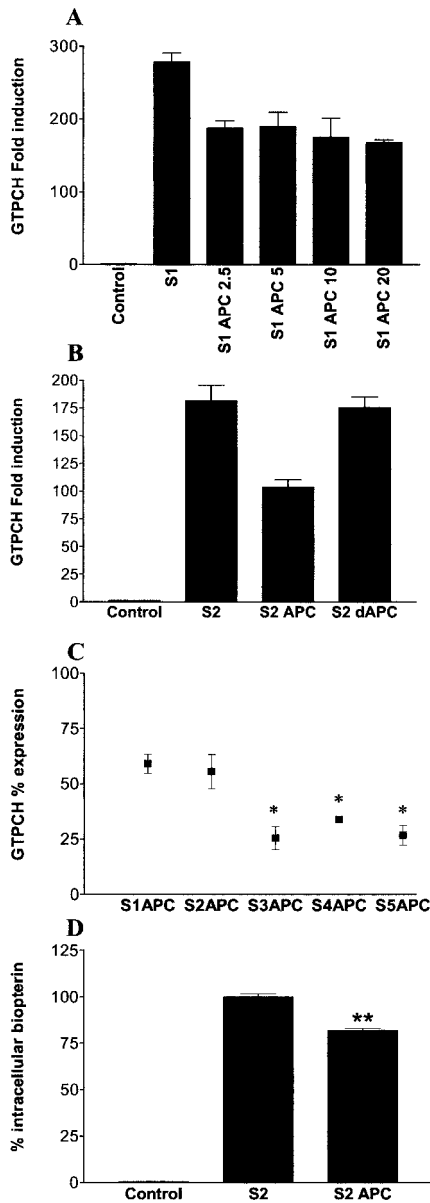


Figure 1. Effect of rhAPC on GTPCH mRNA expression and BH4 synthesis. A, Dose response of rhAPC with the use of HCAECs treated with cytokine mixture S1 (Table 1) for 24 hours alone or in the presence of rhAPC at the doses indicated (2.5, 5, 10, 20 $\mu\text{g}/\text{mL}$). B, Demonstration of the serine protease dependence of the rhAPC effect on GTPCH mRNA expression. HCAECs were stimulated with cytokine mixture S2 (Table 1) alone or in the presence of rhAPC (5 $\mu\text{g}/\text{mL}$) or in the presence of heat-denatured rhAPC (dAPC) (5 $\mu\text{g}/\text{mL}$) for 24 hours. C, Effect of a fixed dose of rhAPC (5 $\mu\text{g}/\text{mL}$) in the presence of different proinflammatory cytokine combinations (S1 to S5; Table 1) for 24 hours. GTPCH mRNA expression was normalized to 100 for the corresponding stimulation without rhAPC. * $P < 0.05$ for HCAECs treated with cytokine mixtures S3 to S5 vs S1 (ANOVA). D, Measurement of intracellular BH4 in HCAECs treated with cytokine mixture S2 (Table 1) alone or in the presence of rhAPC (5 $\mu\text{g}/\text{mL}$) for 48 hours. BH4 was detected as total biopterin on acidic oxidation, as described in Methods. Intracellular biopterin is normalized to 100 in cytokine-stimulated HCAECs. ** $P < 0.01$ for HCAECs stimulated with cytokine mixture S2/rhAPC vs S2. Data are mean \pm SEM (n=4) for A to C; data are mean \pm SD (n=2) for D.

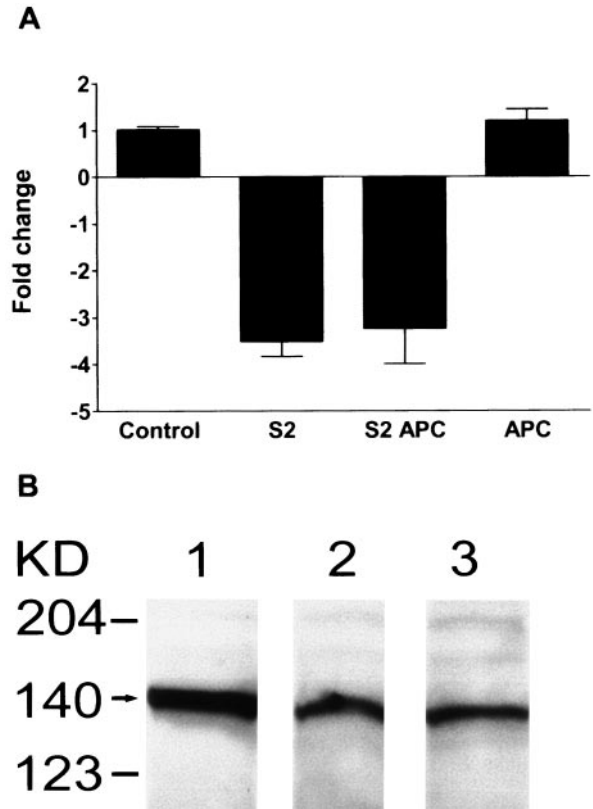


Figure 2. eNOS mRNA (A) and protein (B) expression in HCAECs. A, eNOS mRNA from unstimulated HCAECs was set at 1. HCAECs were stimulated with cytokine mixture S2 (Table 1) alone or in the presence of rhAPC (5 $\mu\text{g}/\text{mL}$) for 24 hours. Data are expressed as mean \pm SEM; n=3. B, Western blot analysis of eNOS protein. HCAECs were treated as described in A for 48 hours. Lane 1, unstimulated cells; lane 2, S2-stimulated cells; lane 3, S2-stimulated cells in the presence of rhAPC.

reduced by $\approx 50\%$ with cytokine mixtures S1 and S2 and by 75% with mixtures S3 to S5. PTPS was not influenced by rhAPC (data not shown). Furthermore, intracellular BH4 was induced by cytokine mixture S2 (Table 1), and rhAPC significantly downregulated BH4 synthesis in inflamed HCAECs (Figure 1D).

Effect of rhAPC on eNOS

Another series of experiments analyzed the effect of rhAPC on eNOS expression by mRNA quantification and Western blot (Figure 2). eNOS mRNA from HCAECs stimulated for 24 hours with cytokine mixture S2 (Table 1) was 3.5-fold downregulated. The addition of rhAPC to these inflamed HCAECs, in concentrations from 5 to 20 $\mu\text{g}/\text{mL}$, did not reverse eNOS mRNA downregulation (Figure 2A). Western blot assays confirmed a 2-fold downregulation of eNOS in stimulated HCAECs (unstimulated, $53.5 \pm 3\%$ adjusted volume density; stimulated, $24 \pm 1\%$ adjusted volume density), and rhAPC had no effect on downregulation of eNOS protein ($23 \pm 1\%$ adjusted volume density; Figure 2B).

Effect of rhAPC on Cytokine, Chemokine, and Adhesion Molecule Gene Expression

In a candidate gene approach, with genes selected from the inflammatory array, we analyzed mRNA expression of IL-6,

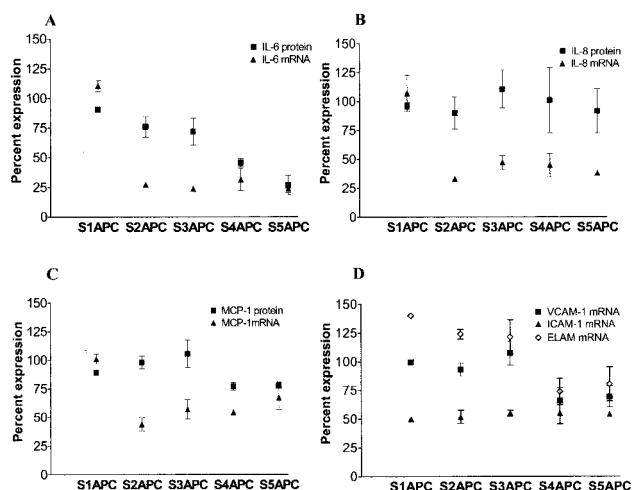


Figure 3. Effect of rhAPC on mRNA and protein expression of proinflammatory cytokines, chemokines, and adhesion molecules in HCAECs stimulated for 24 hours with different inflammatory cytokine combinations (S1 to S5; Table 1) alone or in the presence of a fixed dose of rhAPC (5 μg/mL). In cells stimulated with the corresponding cytokine combinations S1 to S5 alone, expression of the factors analyzed was set to 100%. A, IL-6 mRNA and secreted protein. B, IL-8 mRNA and secreted protein. C, MCP-1 mRNA and secreted protein. D, ELAM, VCAM-1, and ICAM-1 mRNA expression. Data are mean ± SEM; n=4.

IL-8, MCP-1, ELAM, ICAM-1, and VCAM-1 and secreted IL-6, IL-8, and MCP-1 proteins in the tissue culture supernatant. We studied mRNA and protein expression after exposing HCAEC to proinflammatory cytokine mixtures S1 to S5 (Table 1) and confirmed upregulation of all these genes.

As shown in Figure 3A, rhAPC downregulates IL-6 mRNA expression in HCAECs stimulated with cytokine mixtures S2 to S5 ×75%, whereas it had no effect on cells stimulated with cytokine mixture S1. The same effect was also observed on IL-6 protein secretion, although to a lesser extent. IL-6 secretion from cells stimulated with cytokine mixtures S4 and S5 decreased significantly in response to rhAPC, by 50% to 70%, respectively. Similar changes were measured for IL-8 and MCP-1 mRNA expression and protein secretion, in which rhAPC had no effect in S1-treated HCAEC but was effective in cells treated with S2 to S5 (Figure 3B and 3C).

mRNA expression of the adhesion molecules ELAM and VCAM-1 was either not (S1 to S3) or only slightly (S4 and S5) downregulated in response to rhAPC, whereas ICAM-1 mRNA was decreased by 50% in cells stimulated with S1 to S5 (Figure 3D).

Effects of rhAPC on Transcription Factor Activity

In an ELISA-based transcription factor profiling assay, DNA binding activities of the inflammation-related transcription factors ATF2, CREB-1, c-Fos, c-Rel, NFκB p65, NFκB p50, FosB, c-Jun, JunD, Sp-1, and STAT-1 were measured in unstimulated or S4-stimulated HCAECs in the presence or absence of rhAPC for 2 hours. As shown in Figure 4A, the following transcription factors were activated by inflammatory stimuli, and their activation was significantly inhibited by rhAPC: c-Fos, c-Rel, and FosB. RhAPC influenced the activation of FosB most effectively. Even in unstimulated cells, rhAPC diminished activity of c-Fos and FosB (Figure

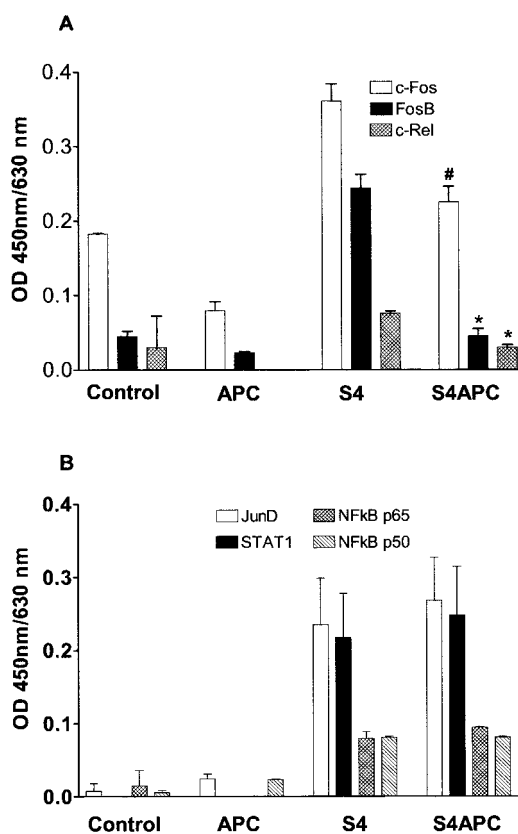


Figure 4. Transcription factor profiling in HCAECs treated with cytokine mixture S4 for 2 hours alone or in the presence of rhAPC (5 μg/mL). A, Activation of transcription factors (c-Fos, FosB, and c-Rel) by cytokine mixture S4 and prevention of their activation by rhAPC in the presence of S4. * $P < 0.001$, # $P < 0.005$. B, Activation of transcription factors (JunD, STAT1, NFκB p65, NFκB p50) by cytokine mixture S4. The presence of rhAPC had no effect on activation by S4. Data are mean ± SEM; n=4.

4A). As shown in Figure 4B, the following transcription factors were activated by inflammatory mixture S4, but their activation was not affected by rhAPC: NFκB p65, NFκB p50, JunD, and STAT-1. Sp-1 and ATF-2 activities were present in HCAECs but were not influenced by cytokines or rhAPC, and CREB-1 and c-Jun activities were not detected in HCAECs (not shown).

Effects of rhAPC on Endothelial Cell Surface Receptors Involved in Inflammation and Coagulation

TF, the initiator of coagulation, was clearly induced by cytokine mixture S3 (Table 1) after 3 hours. After 24-hour exposure to cytokine mixture S3, TF mRNA expression was equal to control HCAEC. The addition of rhAPC did not alter TF mRNA expression in HCAEC. As expected, thrombomodulin mRNA expression was suppressed 2-fold after 3 hours and at least 20-fold after 24-hour exposure to cytokine mixture S3. The addition of rhAPC did not alter thrombomodulin mRNA expression. Endothelial protein C receptor (EPCR) mRNA expression was not altered by exposure to either cytokine mixture S3 or rhAPC. Significant endothelial mRNA expression of protease-activated receptor (PAR) -1, -2, and -3 was detected in untreated cells; however, exposure to the cytokine mixture and/or the addition of rhAPC did not significantly alter their expression (data not shown).

Discussion

To define target genes for rhAPC in inflamed primary vascular endothelium, a gene expression profiling and cluster analysis was performed in HCAECs. Interestingly, only 4.7% of all 8400 analyzed genes were regulated by the most potent cytokine mixture used (S1; Table 1). Of the 400 regulated genes, most genes were upregulated (340), and only 60 were downregulated (Table 2). Gene expression profiling studies performed by others, using HUVECs and only TNF- α as an inflammatory stimulus, found lower percentages of regulated genes.⁸ The greater percentage of regulated genes in our study suggests that the use of combined cytokine mixtures better mimics the pathophysiology of systemic inflammation, in which all these cytokines are present in the system simultaneously. Cytokines in combination are not redundant or additive but highly synergistic.^{9,16} Some genes known to be important for endothelial function and to be regulated by inflammatory stimuli, such as those encoding the enzymes GTPCH,^{9,15} PTPS,⁹ and eNOS,¹⁷ are not caught by the gene expression profiling time frame of 6 hours. Therefore, we analyzed the effect of rhAPC on these genes in direct assays. In previous studies we have shown that BH4 synthesis is upregulated in activated endothelium, as demonstrated by induction of GTPCH and PTPS expression.^{9,10} In the present study we show that rhAPC significantly downregulates GTPCH mRNA expression by 50% to 75%, depending on the potency of the inflammatory mixture used (Figure 1A to 1C). Likewise, the formation of BH4 was significantly reduced by rhAPC in stimulated HCAEC (Figure 1D). Despite PTPS mRNA expression not being affected by rhAPC (data not shown), a direct antiinflammatory action of rhAPC on BH4 synthesis by targeting GTPCH expression is indicated (Figure 1D).

We investigated the endothelial NO-generating enzyme eNOS, which is downregulated by a mixture of inflammatory stimuli due to mRNA degradation.^{17–19} In our system, rhAPC had no influence on eNOS mRNA expression regardless of stimulation used (Figure 2A). This was confirmed at the protein expression level (Figure 2B). That rhAPC has no effect on eNOS expression in our HCAEC system contrasts with eNOS induction by rhAPC reported in unstimulated HUVECs.⁸

The effects of rhAPC were further analyzed with candidate genes selected from our inflammatory gene expression profile. Genes known to be involved in systemic inflammation and sepsis, such as the adhesion molecules ELAM, ICAM-1, and VCAM-1, were chosen. These molecules are known to affect morphology of endothelial cells.²⁰ Indeed, incubation of HCAECs with inflammatory cytokine mixture S1 dramatically changed the morphology from a normal cobblestone appearance to fibroblast-like morphology (not shown). RhAPC modified these changes modestly; however, it was not able to restore cobblestone morphology completely (not shown). This observation gives a visual impression of direct antiinflammatory properties of rhAPC. Consequently, rhAPC downregulated ICAM-1 mRNA expression by 50% regardless of the potency of the inflammatory stimuli (Figure 3D), whereas ELAM and VCAM-1 were only slightly downregulated in cells that received the mildest inflammatory stimuli (S4 and S5; Figure 3A). This could be 1 reason why rhAPC

did not restore cobblestone morphology in HCAECs treated with S1 cytokines.

A remarkable effect of rhAPC was observed on the proinflammatory genes IL-6, IL-8, and MCP-1. RhAPC was not effective on these genes when stimulated with the most potent inflammatory cytokine mixture (S1; Table 1 and Figure 3A to 3C). However, pronounced downregulation of IL-6, IL-8, and MCP-1 mRNA (by 75%) and protein levels was achieved by rhAPC under mild to moderate proinflammatory conditions (S2 to S5; Table 1 and Figure 3A to 3C). This finding not only indicates that rhAPC directly modulates endothelial proinflammatory cytokine production but also suggests that it may be more effective in relatively mild systemic inflammation than in severe sepsis. This question is currently under clinical investigation in a double-blind, randomized, multicenter study (ADDRESS Study; Lilly). In our system rhAPC shows an antiinflammatory effect that depends on the severity of inflammatory activation, without requiring preincubation with rhAPC.⁸

The effect of rhAPC on transcription factor activity revealed a novel insight into transcriptional regulation of these genes. Using an ELISA-based transcription activity assay that is more accurate and sensitive and is highly specific compared with gel shift assays used by others,²¹ we simultaneously measured the effect of rhAPC on 12 different transcription factors. We show for the first time that the inflammation-activated transcription factors c-Fos, FosB, and c-Rel are strongly suppressed in HCAECs by the addition of rhAPC (S4; Table 1 and Figure 4A). However, other inflammation-activated transcription factors such as JunD, STAT-1, NF κ B p50, and NF κ B p65 were not altered by rhAPC regardless of stimulation (Figure 4B).

C-Fos and FosB are members of activator protein-1 (AP-1). AP-1 activity can be induced by several proinflammatory factors.^{22,23} Wang et al²⁴ found that c-Fos and c-Jun, which are not found in HCAECs, directly induce ICAM-1 and MCP-1 in human endothelial cells. In the present study we show for the first time that rhAPC reduces the inflammatory response of HCAECs by downregulating c-Fos activity and thus inhibiting MCP-1 and ICAM-1 expression.

It has been proposed that AP-1 factors can interact with NF κ B to modulate its transactivation activity.^{25,26} C-Rel is a NF κ B family member, and T cells and macrophages from c-Rel knockout mice have reduced cytokine production.²⁷ TF expression is believed to be regulated by interactions between Fos-Jun and c-Rel–NF κ B p65 heterodimers.²⁸ If so, our finding of c-Rel suppression suggests that TF is downregulated by rhAPC; however, we did not observe this with inflamed HCAECs. In vivo, expression of TF, the trigger of the coagulation pathway, has not been detectable on the endothelial cell surface. Therefore, low levels of TF may prevent detection of downregulation of this molecule.

Protein C is a serine protease that inhibits FVa and FVIIIa and thus limits the propagation phase of the clotting process. On binding of circulating thrombin to thrombomodulin, protein C is bound and is proteolytically activated to APC. EPCR, another endothelial receptor, increases the conversion efficiency of protein C to APC on the endothelial surface. Thrombomodulin and EPCR are differentially expressed in

different vascular beds. For instance, thrombomodulin is not expressed in brain endothelial cells, and EPCR is highly expressed in endothelial cells of large vessels.²⁹ We have shown that HCAECs transcribe both endothelial receptors and that EPCR mRNA is not affected by cytokines or rhAPC. Thrombomodulin has previously been described to be down-regulated during inflammation *in vivo*,³⁰ and in our cell-based HCAEC model, thrombomodulin mRNA was strongly down-regulated after exposure to the relatively mild inflammatory cytokine mixture S3 (Table 1). This demonstrates that results of the cell-based model closely represent *in vivo* inflammatory processes.

The antiinflammatory effects of rhAPC are believed to be mediated in a PAR-1- and EPCR-dependent manner.³¹ PAR-1 to PAR-3 are involved in APC signaling, and their mRNAs are expressed in HCAECs. In patients with DIC, protein C and consequently APC are depleted through consumption as a result of low levels of circulating thrombin. Low protein C levels in septic patients are clearly associated with poor survival. Administering rhAPC to these patients not only improved DIC but also improved survival after 28 days.³²

The results presented herein indicate that rhAPC has a direct antiinflammatory effect through regulation of many genes involved in inflammatory processes in the vascular system. Most of the rhAPC-regulated genes are influenced most strongly under rather mild inflammatory conditions. This suggests that rhAPC could be even more effective clinically in patients at high risk of developing severe sepsis, before the onset of symptoms of severe sepsis. Low protein C concentrations have been found in these patients at the onset of fever and before clinical symptoms.³³

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