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Opposite Regulation of Tissue Factor Expression by Calcineurin in Monocytes and Endothelial Cells

Hans Hölscermann, 2* Christine Rascher, ‡ Christian Oelschläger, * Gerald Stapfer, * Andreas Langenstein, * Anne Staubitz, † Ulrich Maus, † Harald Tillmanns, * Holger Bang, 3‡ and Werner Haberbosch*

Tissue factor (TF), a cell membrane-bound glycoprotein that serves as receptor for factor VIIa, plays a key role in the activation of the coagulation cascade (1). When exposed to blood, TF binds to factor VII, and the resulting complex activates factors IX and X, leading to thrombin and fibrin generation. Apart from its primary function in hemostasis, TF participates in other vascular processes including metastasis (2), embryogenesis (3), atherosclerosis (4, 5), and tumor-associated angiogenesis (6).

The expression of TF within the vasculature is strictly regulated and normally not observed under physiological conditions (7). However, TF expression within the vasculature by circulating monocytes as well as vascular endothelial cells (EC) can be induced. This has important implications for intravascular homeostasis and probably plays a significant role in vascular physiology.

The induction of TF expression in monocytes and EC is controlled primarily at the level of transcription (8, 9). With few exceptions, TF expression in both cell types is generally induced by the same agents that elevate the level of cytosolic Ca2++. However, TF expression in other cells is more complex, as it can be regulated at the level of translation and posttranslational modifications (10). The regulation of TF expression in monocytes and EC is controlled by various factors, including nuclear regulatory proteins and transcriptional activators (11, 12).

The expression of TF within the vasculature is strictly regulated and normally not observed under physiological conditions (7). However, TF expression within the vasculature by circulating monocytes as well as vascular endothelial cells (EC) can be induced. This has important implications for intravascular homeostasis and probably plays a significant role in vascular physiology.

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2 Received for publication October 23, 2000. Accepted for publication April 3, 2001.
3 Abbreviations used in this paper: TF, tissue factor; EC, endothelial cell(s); Cn, calcineurin; CsA, cyclosporin A; Cyp, cyclophilin(s); PPIase, peptidyl-prolyl isomerase.

complex Cyp-CsA-Cn. To distinguish between both effects of CsA, we used a nonimmunosuppressive CsA analog, SDZ NIM 811, which binds with the same affinity to Cyp, but does not suppress T cell activation because the resulting drug-Cyp complex does not bind to Cn (14).

The prevailing paradigm regarding the mechanisms of toxic and therapeutic actions of CsA is that it functions to protect against allograft rejection by preventing cell activation, proliferation, and/or cytokine production. A hypothesis, albeit a provocative one, is that some of the CsA effects occur by stimulating the expression of immunomodulating molecules and/or cells. Consistent with this view, recent studies have demonstrated that CsA causes tumor progression by increasing TGF-β expression and enhances TNF-α and IL-12 production in peritoneal macrophages (15, 16). Therefore, we decided to test the gene expression of TF in different cell populations to determine whether CsA might exert its dual action on the same gene product.

We demonstrate that CsA inhibits TF expression in monocytes but enhances it in EC. Our study provides evidence that Cn is the major target for the opposite effects of CsA on TF gene expression and that it elucidates the dual actions of Cn in NF-κB signaling.

Materials and Methods

Cell culture

Monocytes. Human PBMC were isolated from buffy coats obtained from healthy blood donors by Ficoll-Hypaque density gradient centrifugation and further fractionated as described previously (10). The final cultures, suspended at a density of 1 × 10⁶ cells/ml in serum-free culture medium, contained 90–95% monocytes as evidenced by Pappenheim staining and FACs analysis as well as nonspecific esterase staining of cytocentrifuge preparations. Cell viability was >95% as determined by ethidium bromide staining of cell aliquots and subsequent FACs analysis.

EC. EC were isolated from human umbilical veins after collagenase digestion (Collagenase Type II, 175 U/mg; Cell Systems, Kirkland, CA) according to the method of Jaffe et al. (17). All experiments were performed with first passage cells grown to confluence 2 days after seeding. All media and buffers used were assayed for their content of endotoxin by a standard Limulus amebocyte lysate assay (COATEST; Chromogenix, Mölndal, Sweden) and were found to contain <12.5 pg/ml of endotoxin, the lower detection limit of the assay.

Measurement of TF activity

TF activity of cells, lysed by three freeze-thaw cycles, was measured using a one-stage cloting assay as described previously (10, 18). Clotting times were converted to milliliters of TF activity by reference to a standard curve established by serial dilutions of a standard rabbit brain thromboplastin preparation (Sigma, Deisenhofen, Germany). Values are given as mU/10⁶ cells. Factor VII-deficient plasma and nonspecific Ab directed against TF (American Diagnostica, Greenwich, CT) were used to characterize the TF activity.

Determination of TF Ag levels

Endothelial TF Ag was determined by a commercially available ELISA kit (American Dignostica). Monocyte TF expression was examined by flow cytometric analysis. Cells were incubated with or without LPS in the presence or absence of CsA. Cells were incubated for 30 min at 4°C with a FITC-labeled murine anti-human TF mAb for specific detection of TF expression (American Dignostica) or isotype-matched control (BD Biosciences, San Jose, CA). After gating monocytes according to their forward scatter and orthogonal light scatter characteristics, specific TF expression was analyzed in the Fluorescence Two channel (F488/575) of a FACStarplus flow cytometer (BD Biosciences).

Quantification of IL-8 secretion

IL-8 protein levels from culture supernatants of monocytes and EC were measured using commercially available, specific ELISA kits (R&D Systems, Minneapolis, MN). Serial dilutions of the corresponding recombinant cytokine provided standard curves for each individual ELISA plate. Absorbometry measurement was performed at 490 nm on an ELISA reader. The quantification was performed in duplicate.

Analysis of total cellular protein synthesis

Protein synthesis rate was determined by measurement of the incorporation of phenylalanine into cells as described below in detail (10). Briefly, cells were exposed to [3-(U-¹⁴ C)]-phenylalanine (0.1 μCi/ml), and the rate of incorporation into the acid-insoluble cell mass was assayed. Nonradioactive phenylalanine (0.3 mM) was included in the medium to minimize variations in the specific activity of the precursor pool responsible for protein synthesis. DNA content was determined photometrically (19), and the radioactivity was counted. The ratio of incorporated radioactivity (cpm/μg cells) to cellular DNA content served as the parameter for cellular protein synthetic rate.

RNA isolation and RT-PCR

For isolation of total cellular RNA the acid guanidinium thiocyanate-phenol-chloroform method was used as described by Chomczynski and Sacchi (20). Preparation of complementary DNA and subsequent PCR were performed as described before (10). Sequences of intron spanning TF-specific primers were sense 5'-ATCTCGGCCCAACTGTGTA-3’ and antisense 5'-GCTGTCTGTACCTCTCGGT-3’ and for the housekeeping gene β-actin sense 5'-AAAGACCTGTAGCCAACACGAGTGTGCT-3’ and antisense 5'-CGTCAACTCTTCTGGTACACATCTG-3’. Negative controls were performed routinely by running PCR without cDNA to exclude false positive amplification products. The specificity of the obtained TF PCR products was verified by subjecting the related PCR product to automated DNA sequencing (Biometr CTA, Göttingen, Germany) and comparing the resultant cDNA sequence with the published human TF cDNA sequence (21).

EMSA

Nuclear extracts were prepared essentially as described before (10). NF-κB oligonucleotides (5'-AGTTGAGGGGACTTTCCCAGGC-3') were labeled to a specific activity >5 × 10⁵ cpm/μg DNA by end-labeling with [γ³²P]ATP using T4 kinase. NF-κB binding was performed in 10 mM HEPES, pH 7.8, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 4% Ficoll, 100 mM PMSF, 0.02 U calf thymus DNA, and 0.01 U poly(dI·dC) in a total volume of 20 μl. Nuclear extracts were incubated for 30 min at room temperature in the presence of 1 ng labeled oligonucleotide (~50,000 cpm). DNA-protein complexes were separated from the free DNA by electrophoresis on a 4% nonacylaminol gel in 1/4× Tris-boric acid-EDTA (TBE) buffer and autoradiographed. Specificity of binding was ascertained by competition with a 160-fold molar excess of cold NF-κB consensus oligonucleotides. Band intensities were quantified by densitometric analysis.

Western blot analysis

For Western blot analysis, 10 μg of cytoplasmatic protein extract was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed using rabbit polyclonal Ab specific for IκBα (c-21, 1/3000 dilution, 1-h incubation; Santa Cruz Bio- technology, Santa Cruz, CA) and secondary goat anti-rabbit Ab (sc-2004, 1/600 dilution, 1-h incubation; Santa Cruz Biotechnology). For detecting the serine 23-phosphorylated IκBα, rabbit anti-human IκBα Ab (9241S; New England Biolabs, Beverly, MA) was used at a 1/1000 dilution. Immunoreactive proteins were visualized by the use of an ECL kit (Amer sham, Arlington Heights, IL).

Assay of prolyl isomerase activity

As described earlier, a chymotrypsin-coupled assay was used to measure the prolyl isomerase activity. Suc-Ala-Leu-Pro-Phe-4-nitroanilide was used as the assay substrate (22). The reaction was initiated by adding 6 μl of the substrate solution to the reaction mixture containing HEPES buffer, chymotrypsin, and either cell extracts or control solvent vehicle. First-order rate kinetics were observed with a rate constant of kobs = kₚ + k₋ₚ, and kobs = kₚ + k₋ₚ/kₚ, where kₚ is the rate constant of the uncatalyzed cis-to-trans interconversion, and k₋ₚ is the observed first-order rate constant in the case of PLase catalysis. Cell extracts from monocytes and EC were prepared as described for the Cn assay. For comparing the prolyl isomerase activities in the extracts of the cells, the arbitrary unit AU = (k₋ₚ/kₚ) × 1 per milligram protein was used. For inhibition experiments, 2 μl of ethanol, CnA, or SDZ NIM 811 (stock solution-prepared ethanol) were added 15 min before starting the reaction.

Characterization of Cyp isoforms

Characterization and purification of Cyp isoforms were performed with modifications of our earlier described procedure (23). Cell extract was
FIGURE 1. Effect of CsA on TF induction in monocytes and EC. A, TF activity. Cells were stimulated with LPS (10 μg/ml; 8 h) in the presence or absence of varying concentrations of CsA (0–5 μmol/L). Values of TF activity of cells (determined by clotting assay) are shown as mean ± SD of three different experiments, each performed as triplicate determinations. * p < 0.05 compared with cells stimulated in the absence of CsA. B, TF expression. Down-regulation of TF expression by CsA in monocytes. Cells were either left unstimulated (gray histogram of low red fluorescence intensity) or stimulated (LPS, 10 μg/ml, 8 h) in the absence (closed line, increased red fluorescence intensity) or presence of CsA (5 μmol/L; dashed line, low red fluorescence intensity) followed by flow cytometric analysis of TF expression. The x-axis gives red fluorescence intensity (F488/575) and the y-axis gives cell numbers (plotted on linear scale). Up-regulation of TF expression by CsA in EC: EC were either left unstimulated or stimulated (TNF-α, 40 ng/ml, 8 h) in the absence or presence of CsA. Subsequently, TF Ag measured in whole cell lysates by ELISA as outlined in Materials and Methods. * p < 0.05 compared with cells stimulated in the absence of CsA. C, Effect of CsA on LPS-induced IL-8 production in monocytes and EC. Following stimulation of cells with LPS (10 μg/ml; 8 h) in the presence or absence of CsA (5 μmol/L), IL-8 protein levels from culture supernatants were measured by ELISA. Data represent mean ± SD of triplicate cultures for each experimental condition, with IL-8 assays performed in duplicate. * p < 0.05 compared with cells stimulated in the absence of CsA. # p < 0.005 compared with cells stimulated in the absence of CsA.
prepared as described for the Cn assay. The supernatant was incubated at 40°C for 8 h with 20 μl of a CsA affinity resin, containing t-ALA-(3-amino)-8-cyclosporine (provided by Sandor, Basel, Switzerland) coupled to CNBr-activated Sepharose (Amersham, Pharmacia Biotech, Piscataway, NJ). After washing, Cyp isoforms were eluted with 10 μM of CsA in 50 mM HEPES buffer, pH 7.5, and analyzed on a 10% bis-Tris-NCPAGE gel in a MES running buffer (Invitrogen, San Diego, CA). To evaluate the amount of the eluted Cyp isoforms, an image of the SDS-PAGE was recorded with a cooled charge-coupled device camera (DIANA; Raytest, Straubenhardt, Germany) and relative quantities were estimated with Aida software. To identify the eluted proteins, Western blot analysis was performed with our earlier described polyclonal anti-Cyp A Ab (23) or a polyclonal anti-Cyp40 Ab (Alexis, Grünberg, Germany) and visualized by using the ECL Western blotting detection kit (Amersham Pharmacia Biotech). Quantification of bound Cyp A and Cyp40 was performed by scanning densitometry.

**Immunounoassay for Cyp**

A quantitative sandwich capture ELISA was developed for CypA as follows. Flat-bottom microtiter plates (Costar, Cambridge, MA) were coated with 100 μl/well (5–50 μg/ml) murine anti-Cyp7 F1 mAb (23) in a coating buffer and blocked with PBS containing 1% BSA. Undiluted (2 μg/ml) and serial dilutions of the standard Ag, human Cyp A (23), were added to the wells. The supernatant of homogenized monocytes or EC was added at a protein concentration of 100-2000 μg/ml (diluted in PBS/1% BSA). After a 2-h incubation at 37°C, bound Cyp were detected by a rabbit anti-Cyp A antiserum (23), followed by goat anti-rabbit IgG-coupled HRP (1:5000; Dianova, Hamburg, Germany). The substrate tetramethylbenzidine (Sigma, St. Louis, MO) was used, and the absorbance at 450 nm was measured. The experiment was repeated with two different preparations of each cell line.

A semiquantitative analysis of Cyp40 in monocytes and EC was performed by Western blot, using recombinant Cyp40 as standard Ag (2–200 ng protein/lane) to calibrate the Western blot signals and a polyclonal anti-Cyp40 antiserum (Alexis). Cells extracts containing either proteins from monocytes or EC (200 μg protein and serial dilutions thereof) were fractionated on 4–12% NuPAGE polyacrylamide gels (Invitrogen) and electrophoretically transferred overnight in NOVEX transfer buffer (Invitrogen) at 100 mA and at 4°C onto ECL-Hybond nitrocellulose membranes (Amersham). Cyp40 was detected by incubation with anti-Cyp40. Anti-rabbit-conjugated HRP (1:2000) was used as secondary Ab (Dianova), and the blot was developed using the ECL Western blotting detection kit (Amersham). All quantitation was performed using an Advanced Imaging System (DIANA, Raytest). AIDA Image Quant software (Raytest) as a modular software package for evaluation and documentation of the images was used to quantify each band within a given blot with appropriate background subtraction. The calibration standard recombinant Cyp40 was used by AIDA to convert the relative intensity integrals to absolute measurements of μg protein. Each experiment was repeated twice.

**Cn assay**

Cn activity was assayed as described with minor modifications (24). The RII peptide (DLYVPGPRDRKSVAEE; Biomol, Hamburg, Germany) was labeled to ~600 cm³/μmol and purified on a RP-C2 clean up extraction column (Amchrom, Sulzbach, Germany) as previously described (23). The peptide was eluted with 80% acetonitrile and freeze-dried, and reconstituted samples were stored in deionized water at ~80°C. Cells were evaluated for their ability to dephosphorylate the RII peptide in the presence of okadaic acid (Biomol, Hamburg, Germany), a phosphatase type 1 and 2A inhibitor (25). Background phosphatase 2C activity (CsA- and okadaic acid-resistant activity) was determined and subtracted from each sample, with the remaining peptide being the presence and absence of excess added CsA. Cells were homogenized in lysis buffer, and a mixture of protease inhibitors was added (PharMingen, San Diego, CA). Extracts were microcentrifugated, and supernatants were transferred to fresh tubes. Protein content was quantified by the Bradford method.

Assays were performed in duplicate at 30°C in 50-μl assay buffer and 0.1-μM calmodulin (Sigma). Where indicated, the following ordered additions were made in the assay: 5 mM β-mercaptoethanol, 300 nM okadaic acid, 20 nM to 2 μM CsA or its solvent ethanol. The reaction was initiated by the addition of [32P]RII (5 μM). After 30 min at 30°C, the extent of [32P]RII dephosphorylation was analyzed after addition of 0.5-ml 0.1 M potassium phosphate with 5% TCA. Dowex cation exchange resin (AG50W-X4H+ form, 400 mesh; Bio-Rad, Hercules, CA) was added. The released [32P]phosphate containing supernatant was measured by scintillating counting, CsA-resistant phosphatase values were subtracted, and Cn activity was expressed as picomoles of released 32P per minute per milligram of lysate proteins.

**Statistics**

Experiments were performed in triplicate. Data are shown as means ± SD. Statistic significance was estimated with one-way ANOVA with pairwise contrasts by Scheffé. Differences were assumed to be statistically significant when p values were <0.05.

**Results**

CsA differentially regulates TF production in monocytes and EC

Fig. 1 illustrates the distinct effects of CsA on the induction of TF expression observed in monocytes and EC. Whereas treatment with CsA led to a dose-dependent inhibition of TF expression in monocytes, simultaneous treatment with CsA augmented the expression of TF in EC. The enhancing effect in EC was observed with concentrations of CsA that entirely abrogated the TF response to LPS in monocytes. The effect of CsA on inducible functional TF clotting activity (Fig. 1A) was paralleled by a decrease (monocytes) or an increase (EC) in immunologically detectable TF protein expression (Fig. 1B). Similar to its effect on TF expression, CsA stimulates (in EC) or antagonizes (in monocytes) the induction of IL-8 (Fig. 1C). The inhibitory effect of CsA on TF or IL-8 induction in monocytes was not due to an overall reduction of the total protein-synthetic rate in the presence of CsA as demonstrated by analysis of 14Cphenylalanine uptake by cells treated with CsA vs control cells (107 ± 19% of control for 0.1 μmol/L CsA; 99 ± 20% of control for 5 μmol/L CsA) or due to any toxic effect on cells, as evidenced by a viability test of cells by ethidium bromide staining and subsequent FACS analysis.

**Effect of CsA on PPlase activities in monocytes and EC**

The above studies suggested that CsA might modulate TF expression either through its interference with the PPlase activity of Cyp or by inhibiting CN via a preformed CsA-Cyp complex. Therefore, the cis-trans isomerization of the test peptide succinyl-Ala-Leu-Pro-Phe-4-nitroanilide was used in a coupled assay for measuring the isomerase activity of Cyp in the cells (Table I). The data show that both cells had similar amounts of overall PPlase activity. When CsA (up to 3 μM), known to bind and inhibit Cyp, was added to the cell lysates the PPlase activity of the Cyp-like proteins was strongly inhibited to a similar level, with no significant differences in the inhibition between monocytes and EC.

Additionally we addressed the question of whether there is a difference in the levels of Cyp isomerase expression between EC and monocytes, making the former cells resistant to CsA. By using an affinity purification on a CsA resin, we characterized the different Cyp isoforms in the corresponding cells. As shown in Fig. 2, the pattern of Cyp isoforms that bind to the CsA affinity resin did not differ among monocytes and EC (Fig. 2A), apart from an higher bound of level bound CypA and Cyp40 in monocytes as in EC (Fig. 2B). Immunoblots with whole cell lysates demonstrated similar levels of CypA and Cyp40 expression (Fig. 2C). For the determination of CypA concentrations in cell extracts of monocytes and EC a sandwich ELISA using Cyp A mAb was developed as described in Materials and Methods. The concentration of CypA was in the range of 1.7–2.4 μg/ml total protein without a significant difference between monocytes and EC (Fig. 2D). Moreover, comparable amounts of Cyp40 were present in total cell extracts of monocytes and EC as shown by use of a semiquantitative immunoblot. Using the different methods, a severalfold higher expression level for CypA than for Cyp40 was found in both cells, comparable with other human cell lines and tissues (23). These results indicate that the difference in binding to the CsA resin was possibly attributable to a difference in intracellular interactions of the Cyp from both cells, which are competitive to CsA binding. Surprisingly, this
intracellular situation seems not be detectable by the commonly used PPIase activity assays.

Finally, to determine whether the PPIase activity of Cyp is related to the modulatory action of CsA on TF expression in monocytes and EC, additional experiments were performed with a non-immunosuppressant CsA analog, SDZ NIM 811, which inhibits Cyp PPIase activity, but not Cn. Inhibition of the PPIase activity by SDZ NIM811 did not affect the induction of TF activity in either monocytes or EC (Fig. 3).

Cn inhibitors oppositely modulate the induction of TF in monocytes and EC

The above observations led to the prediction that CsA may effect TF induction in monocytes and EC by selectively interacting with certain intracellular signal transduction pathways besides inhibition of PPIase activity. The induction of TF expression in monocytes and EC by various agonists has the common requirement for an increase in the concentration of intracellular Ca\(^{2+}\). One potential mediator of these Ca\(^{2+}\)-dependent signaling pathways is Cn. This Ca\(^{2+}\)/calmodulin-dependent protein phosphatase is known to be the major target of CsA in T cells. The following observation demonstrates the crucial role of Cn in TF induction in monocytes and EC. As shown in Table II, Cn is functionally present in both cells in comparable concentrations. The inhibition constant for Cn by CsA is the same for both cells and correlates well with the observed biological effects of TF expression. Moreover, cypermethrin, a further potent Cn inhibitor (26), regulated TF induction in monocytes and EC in a similar manner as CsA by preventing TF induction in monocytes but augmenting it in EC (Fig. 4, upper panel). The opposite effect of Cn inhibition on TF induction was not limited to endotoxin stimulation, but was also observed when other agonists were used to induce TF. In contrast, inhibition of Cn by cypermethrin augmented TF induction in EC (Fig. 4, lower panel). In both cells, TF expression was insensitive to treatment with okadaic acid or tautomycin when added to cells in concentrations known to inhibit the serine/threonine phosphatases 1 and 2A (data not shown) (27, 28).

**FIGURE 2.** Monocytes and EC express the same pattern of binding proteins for CsA. A, Cell lysates from monocytes (lane 1) and EC (lane 2) were loaded onto a CsA affinity column, extensively washed, and eluted with CsA. The eluted proteins were analyzed by SDS-PAGE and Coomassie blue staining. B, Quantitation of the relative level of Cyp that bind to the CsA beads was performed by scanning densitometry using Aida image software. The results are given in arbitrary units for CypA (right) and Cyp40 (left) in monocytes (a) and EC (b). C, Assay for CypA and Cyp40 by Western blot. Equal quantities of protein from the whole cell extracts from A were separated by electrophoresis, transferred to membranes, and probed with the indicated antisera. D, Quantitative analysis of Cyp40 and CypA expression in monocytes (a) and EC (b) was performed by a sandwich capture ELISA for CypA (left) and by scanning densitometry of the Cyp40 immunoblot (right), respectively. The data set is the average of two independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PPIase Activity (AU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monocytes</td>
</tr>
<tr>
<td>Solvent</td>
<td>171 ± 14</td>
</tr>
<tr>
<td>CsA (1 μM)</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>CsA (3 μM)</td>
<td>61 ± 5</td>
</tr>
<tr>
<td>SDZ NIM811 (1 μM)</td>
<td>75 ± 8</td>
</tr>
<tr>
<td>SDZ NIM811 (3 μM)</td>
<td>71 ± 11</td>
</tr>
</tbody>
</table>

*PPIase activities were measured in cell homogenates of monocytes and EC, respectively, toward Suc-Ala-Leu-Pro-Phe-pNA with the isomer-specific proteolysis as described in Materials and Methods. Means ± SEM were calculated from at least triplicate determinations of two independent cell preparations.
Cn oppositely regulates the transcriptional activation of the TF gene in monocytes and EC

To study whether Cn might be a regulatory mediator for the transcriptional activation of the TF gene, RT-PCR was performed with primers specific for the TF cDNA. Consistent with the results obtained on TF protein expression, simultaneous inhibition of Cn (by either CsA or cypermethrin) resulted in reduced levels of TF mRNA in monocytes. In contrast, TF mRNA expression was induced by simultaneous Cn inhibition in EC (Fig. 5).

The opposite effects of Cn inhibitors on TF gene transcription in monocytes and EC led us to investigate whether Cn affects the signal cascade before gene transcription. In both cell lines, TF gene transcription is known to be controlled by the transcription factor NF-κB, which is sequestered in the cytoplasm by inhibitory proteins, such as IκBα (29). As shown in Fig. 6, degradation of IκBα followed by nuclear import of NF-κB were observed with similar kinetics in response to LPS in both cells. However, whereas simultaneous inhibition of Cn reduces NF-κB activity in monocytes, Cn inhibitors enhanced NF-κB binding activity in EC (Fig. 7, upper panels).

Table II.  Cn phosphatase activity in monocytes and EC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cn Activity (pmol/min/mg protein)</th>
<th>Monocytes</th>
<th>EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td></td>
<td>27.8 ± 4.3</td>
<td>34.1 ± 3.1</td>
</tr>
<tr>
<td>5 mM EGTA</td>
<td></td>
<td>13.7 ± 2.3</td>
<td>19.5 ± 4.3</td>
</tr>
<tr>
<td>500 nM okaidic acid (OA)</td>
<td></td>
<td>7.1 ± 1.5</td>
<td>8.3 ± 1.2</td>
</tr>
<tr>
<td>OA + 0.1 μM CsA</td>
<td></td>
<td>6.7 ± 0.8</td>
<td>7.8 ± 1.7</td>
</tr>
<tr>
<td>OA + 1 μM CsA</td>
<td></td>
<td>2.1 ± 0.9</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>OA + 5 μM CsA</td>
<td></td>
<td>1.5 ± 0.7</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>OA + 0.1 μM cypermethrin</td>
<td></td>
<td>1.7 ± 0.4</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>OA + 5 μM SDZ NIM811</td>
<td></td>
<td>7.3 ± 1.9</td>
<td>8.2 ± 1.7</td>
</tr>
</tbody>
</table>

*Phosphatases PP1 and PP2A comprise the majority of phosphatase activity in both cell types supported by calcium substitution with EGTA. The remaining okaidic acid-resistant phosphatase activity is due to Cn (~70–85%) and PP2C (15–30%). All values are means ± SEM (n = 3) from two different cell preparations of each cell type.

Agonists such as LPS induce import of NF-κB by phosphorylation of IκBα, which is then ubiquinated and rapidly degraded by the 26S proteasome (30). Therefore, to examine the fate of IκBα upon inhibition of Cn, IκBα degradation and phosphorylation in the presence of CsA was analyzed by immunoblotting. Fig. 7 shows that CsA prevents IκBα degradation in monocytes. Similar results were observed by Cn inhibition with cypermethrin. In contrast, simultaneous inhibition of Cn increases degradation of IκBα upon stimulation in EC (Fig. 7, middle panels). As monitored by immunoblot analysis using Abs specific for the Ser32-phosphorylated form of IκBα, the loss of cytoplasmatic IκBα was consistent with the occurrence of phosphorylated IκBα 5–10 min upon stimulation in monocytes and EC. However, when stimulation of cells was performed in the presence of the Cn inhibitor CsA, reduced IκBα phosphorylation was detected in monocytes, whereas inhibition of Cn resulted in an enhanced IκBα phosphorylation in EC (Fig. 7, lower panels). These results show that Cn differentially controls NF-κB transactivation in monocytes and EC at the level of IκBα proteolysis by either enhancing (in monocytes) or suppressing (in EC) IκBα phosphorylation.

Discussion

TF, a small transmembraneous glycoprotein, is the major cellular initiator of blood coagulation. The constitutive production of extravascular TF by fibroblasts, smooth muscle cells, and other cells from various organs has been proposed to create a hemostatic “envelope” prepared for immediate activation of the coagulation cascade in case of vascular injury (7), a mechanism which is essential for survival of multicellular organisms. However, in the last few years it has become increasingly clear that TF has important additional functions apart from its role in blood coagulation. Recent studies on transgenic and TF knockout mice have demonstrated the involvement of TF in angiogenesis (6), embryogenesis (3), metastasis (2), and atherosclerosis (4). The role of TF in these processes has drawn attention to cells able to inducibly express TF within the vasculature, i.e., circulating blood monocytes and vascular EC.

The regulation of TF synthesis in monocytes and EC, normally transcriptionally silent, would appear to be a critical step in the
control of blood vessel development, tumor growth, and cell proliferation after vessel wall injury.

In monocytes and EC, the induction of TF is under transcriptional control. With few exceptions, TF expression in both cells is generally induced by the same agents that elevate the level of cytosolic Ca\(^{2+}\) and whose intracellular signaling pathways converge to activate the transcription factor NF-κB, allowing translocation of NF-κB/Rel complexes to the nucleus (8, 9). In the nucleus, these complexes bind to their target sites in the TF gene promoter to regulate the induction of TF gene transcription. Although the regulatory DNA elements that control TF gene transcription are well characterized, the signaling pathways downstream from intracellular Ca\(^{2+}\) mobilization leading to NF-κB activation were not yet established.

One potential mediator of such Ca\(^{2+}\)-triggered events is Cn, a Ca\(^{2+}\)/calmodulin-dependent serine/threonine phosphatase that is expressed ubiquitously in eukaryotic cells (reviewed in Ref. 31). Cn, also known as phosphatase 2B (32), has primarily been established as the key enzyme in the Ca\(^{2+}\)-dependent NF-AT transactivation in T cells. The pivotal role of Cn in T cell signaling was first appreciated by its identification as the target enzyme of CsA, which selectively inhibits the phosphatase activity of Cn when complexed to its intracellular binding protein, Cyp. We have demonstrated earlier that CsA interferes with the signal transduction pathway leading to TF expression in monocytes (10). CsA has proved to be a valuable reagent for probing signal transduction pathways, as it is known to act at a step distal to the cell membrane receptors and downstream of the second messenger Ca\(^{2+}\), but proximal to late signaling events (33). In this study, using two different Cn inhibitors, CsA and the potent phosphatase 2B inhibitor cypermethrin as probes, we elucidated the participation of Cn in the regulation of NF-κB transcriptional activity in monocytes and EC.

Inhibition of Cn by either CsA or cypermethrin exerted opposite effects on the induction of NF-κB in monocytes and EC. Whereas activation of NF-κB was blocked by cypermethrin as well as CsA in monocytes, both agents enhanced the agonist-induced NF-κB binding and led to an increased TF protein and mRNA expression in EC. The observation that both CsA and cypermethrin interfere with the signal transduction pathway leading to TF expression in monocytes (10) and EC (33) was confirmed in this study.

**FIGURE 4.** Effect of inhibition of Cn on TF production in monocytes and EC. Cypermethrin, a further potent inhibitor of Cn, regulated agonist-triggered TF expression in a similar manner as CsA by decreasing TF induction in monocytes (upper panel) but enhancing it in EC (lower panel). To further investigate whether the opposite effect of Cn inhibition was limited to LPS stimulation (10 μg/ml), the effect of cypermethrin (1 nmol/L) was also tested in the presence of TNF-α (40 ng/ml) and the calcium ionophore ionomycin (1 μmol/L), respectively, additional agonists known to induce TF in both cell types. TF values are shown as means ± SD of three different experiments, each performed as triplicate determinations. *, p < 0.05 compared with cells stimulated in the absence of cypermethrin.

**FIGURE 5.** Effect of inhibition of Cn on TF mRNA expression in monocytes and EC. Cells were incubated with ionomycin (1 μmol/L) alone or in combination with cypermethrin or CsA, respectively, as indicated. Cells were harvested following 3–4 h of stimulation, and RT-PCR was performed with primers specific for the TF and β-actin gene. A representative photograph of PCR-amplified products of TF and β-actin mRNA is given. Similar results were obtained in three independent experiments.

**FIGURE 6.** Time course of LPS-induced activation of NF-κB and degradation of IκBα in monocytes and EC. Cells were either not stimulated or treated with LPS (10 μg/ml) for the times indicated. **Upper panels,** Nuclear extracts were prepared and assayed by EMSA using a 32P-labeled NF-κB consensus oligonucleotide. Specific NF-κB complexes are indicated by an arrow. Three independent experiments with each cell type showed similar results. **Lower panels,** Cytosplasmatic proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with polyclonal Abs against IκBα. A representative immunoblot of three different experiments is shown.
with a signal transduction pathway that is critical for NF-κB activation in monocytes and EC strongly suggests that the phosphatase Cn may be the target enzyme of this action.

The inverse effect of Cn on the activation of NF-κB observed in both cell types was accurately documented not to be related to differences in pharmacological properties of monocytes and EC. Both cells contained comparable levels of Cn, as defined by activity measurements in this study, and incubation of cells with both compounds resulted in a similar, dose-dependent inhibition of Cn phosphatase activity in both monocytes and EC. The different susceptibility of monocytes and EC to CsA with respect to NF-κB activation was also demonstrated not to result from a modified CsA binding capacity in both cell types. The level of Cyp, as well as the pattern of Cyp isoforms, do not differ among monocytes and EC. In addition, the concentration of Cys that inhibits the intrinsic PPIase activity of Cyp was identical in both cell lines. In experiments using the nonimmunosuppressive CsA analog SDZ NIM 811, a compound that blocks the PPIase activity of Cyp but does not target Cn, it was also excluded that the modulatory action of CsA might be related to the inhibition of the PPIase activity of Cyp. These data indicate that 1) two different signaling mediators, the phosphatase Cn in monocytes and an as yet unidentified enzyme in EC, control NF-κB activation, and 2) Cn cell specificity is implicated in the activation of NF-κB as it induces NF-κB activation in monocytes but suppresses NF-κB activation in EC. Our observations are consistent with previous studies proposing a role for Cn in generating transcriptionally active NF-κB and they confirm the presumption that the ability of Cn to participate in NF-κB transactivation of genes is not cell specific (15, 34, 35).

Cn must be considered as an essential intermediate in the signaling pathway that differentially controls NF-κB activation in vascular cells. How then is Cn involved in NF-κB regulation? NF-κB is sequestered in the cytoplasm as an inactive complex associated with its inhibitor IκBα (36). Western blot analysis in this study, with mAbs recognizing IκBα, revealed that the agonist-induced degradation of IκBα can be blocked by inhibiting the phosphatase activity of Cn in monocytes. Inhibition of Cn prevented IκBα phosphorylation and degradation in monocytes in the same manner as has been shown for phorbol ester and ionomycin treatment of Jurkat T leukemia cells (37). Our results also confirm previous studies proposing Cn participation in NF-κB induction by indirect stimulation of IκBα inactivation (38, 39). In contrast, simultaneous inhibition of Cn in EC further enhances inducible IκBα phosphorylation. A similar negative regulatory role of Cn in expression of various genes has also been observed in other cell types (16, 40–42). In the absence of specific stimuli, inhibition of Cn in EC had no demonstrable effect on IκBα phosphorylation. Thus, Cn may primarily function to attenuate the endothelial NF-κB response when cells are challenged by exogenous stimuli. Taken together, these results demonstrate that Cn differentially modulates NF-κB transactivation in monocytes and EC on the level of IκBα by either enhancing (in monocytes) or suppressing (in EC) IκBα phosphorylation.

The precise mechanism whereby Cn participates in IκBα phosphorylation in EC remains to be elucidated. The synergy in IκBα phosphorylation by CsA could be mediated by at least two processes. One possibility is that IκBα is a direct target of Cn, and that maintenance of phosphorylated IκBα in the presence of CsA in EC directly results from inhibition of the phosphatase activity of Cn. Rescue of IκBα from degradation by serine dephosphorylation has been reported (43), but so far linked to Cn only in astrocytes (44). The second possibility, suggested by the findings of other investigators, is that Cn is required for effective activation of the IκBα kinase complex, which is responsible for IκBα phosphorylation. In Jurkat and primary human T lymphocytes, Cn has been shown to be necessary for phosphorylation of IκBα, as inhibition of Cn (similar to our results in EC) reverses the activation of the IκBα kinase complex and IκBα phosphorylation in vivo (35). As signal-induced phosphorylation of IκBα is a multistep process in which site-specific phosphorylations of upstream regulators are the main molecular events, further phosphorylated Cn substrates may exist in the upstream signaling pathway. The distinct behavior of IκBα in monocytes and EC following inhibition of Cn substrates may also be related to the existence of cell-specific Cn substrates. Future information about the direct targets of Cn will serve to better identify to which extent Cn functions to modulate NF-κB activation in vascular cells.

In summary, these studies indicate that 1) Cn activity is a drug-sensitive step controlling NF-κB transactivation and 2) Cn differentially regulates the NF-κB controlled signaling leading to TF gene transcription in monocytes and EC. These results may have important implications for new research on vascular treatment strategies for NF-κB-mediated clinical disorders, such as inflammatory and immunologic diseases, atherosclerosis, or cancer, in which targeting of specific components of NF-κB activation appears to be advantageous.

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References


