Angiotensin-Converting Enzyme Inhibitors Downregulate Tissue Factor Synthesis in Monocytes

Emanuela Napoletane, Angelomaria Di Santo, Marina Camera, Elena Tremoli, Roberto Lorenzet

Abstract—Angiotensin-converting enzyme (ACE) inhibitors reduce the risk of recurrent myocardial infarction in patients with left ventricular dysfunction. Tissue factor (TF), the initiator of blood coagulation, plays a pivotal role in arterial thrombosis that occurs after atherosclerotic plaque fissuring. Because monocytes synthesize TF and contain several components of the renin-angiotensin system, we investigated the possibility that ACE inhibitors could modulate monocyte TF expression. Mononuclear leukocytes from healthy volunteers were incubated with endotoxin in the presence or absence of different ACE inhibitors. Captopril reduced TF expression in endotoxin-stimulated mononuclear leukocytes, as measured by a 1-stage clotting assay and ELISA analysis, by \approx 60\%. The effect was dose-dependent and was attributable to ACE inhibition, given that other ACE inhibitors, such as idrapril or fosinopril, and losartan, an antagonist of the angiotensin II AT1 receptor, caused a comparable reduction in TF activity. Reverse transcriptase–polymerase chain reaction indicated that endotoxin-mediated increased levels of TF mRNA were inhibited by ACE inhibitors. Moreover, endotoxin-induced nuclear factor-\kappa B translocation to the promoter region of the gene encoding for TF was markedly inhibited by captopril. The finding that ACE inhibitors and angiotensin II AT1 antagonists can potentially modulate TF expression by mononuclear cells has important biological and therapeutic implications for the evolution of thrombi. Our results suggest that the anti-ischemic effect of these drugs might be explained, at least in part, by their ability to reduce TF expression in monocytes. (Circ Res. 2000;86:139-143.)

Key Words: angiotensin ■ coagulation ■ leukocyte

Angiotensin-converting enzyme (ACE), a component of the renin-angiotensin system, is considered to play a central role in the maintenance of blood pressure and the regulation of fluid and electrolyte homeostasis.\(^1\) Recently, ACE inhibitors have been proposed to exert anti-ischemic effects, reducing the risk of recurrent myocardial infarction in patients with left ventricular dysfunction. In particular, ACE inhibitors cause regression of left ventricular hypertrophy.\(^2\)–\(^4\) Although most of the effects of ACE inhibitors are generally linked to their anti-hypertensive action, numerous reports suggest alternative mechanisms related to the atherosclerotic process. The renin-angiotensin system has been implicated in the proliferative response of the vascular wall after arterial injury,\(^5\) in the biosynthesis of plasminogen activator inhibitor type 1 and tissue plasminogen activator,\(^6\) and in the synthesis of proto-oncogenes that are linked to the adaptive process of cardiovascular hypertrophy.\(^6\) Tissue factor (TF) is a transmembrane glycoprotein that, on binding to coagulation factor VII and its active form VIIa, forms the cell surface complex responsible for the coagulation sequence, which lead to fibrin formation.\(^7\) TF is constitutively present on the surface of nonvascular cells, providing the body with a defense mechanism apt to stop the loss of blood in case of tissue injury. Although normally not present on cells in contact with blood, monocytes and endothelial cells can be induced by several agonists to synthesize and express TF on their membranes.\(^8\)–\(^10\)

TF gene expression is regulated principally at the level of transcription.\(^11\) In endotoxin (bacterial lipopolysaccharide)−stimulated monocytes, activation of the TF gene induces the translocation to the nucleus of c-Rel/p65 heterodimers, which belong to the family of transacting factors nuclear factor (NF)−\kappa B, where they bind to a putative \kappa B site in the TF promoter.\(^12\)

The expression of TF on monocyte membrane is potentially involved in thrombus formation in a variety of pathological conditions such as immune-inflammatory diseases,\(^13\) septic shock,\(^14\) and cancer.\(^15\) Recently, monocyte TF has been proposed as the main protein responsible for the thrombotic complications of atherosclerosis. The presence of
mRNA coding for TF has been reported in macrophage foam cells and monocytes adjacent to the cholesterol clefts in atherosclerotic plaques from patients undergoing carotid endarterectomy.\textsuperscript{21} Moreover, macrophages isolated from carotid atherosclerotic plaques were found to express a marked procoagulant activity.\textsuperscript{22} In addition, a role for monocyte TF has been proposed in unstable coronary syndromes.\textsuperscript{23,24} In particular, increased expression of TF was found in atherectomy specimens from patients with unstable angina\textsuperscript{25–27} or myocardial infarction.\textsuperscript{27}

To gain knowledge of the potential mechanism of the antithrombotic properties of ACE inhibitors, we studied their effect in the synthesis and expression of TF by human monocytes. We now offer the first direct evidence that ACE inhibitors downregulate TF expression in endotoxin-stimulated human monocytes and that this effect is mimicked by the angiotensin II AT\textsubscript{1} receptor antagonist losartan.

**Materials and Methods**

**Cell Isolation and Culture**

Mononuclear cells were obtained from blood from healthy volunteers as previously described and were resuspended at a final concentration of \(3 \times 10^6\) cells/mL.\textsuperscript{26} The monocytes in this population were 25% to 30%, as assessed by nonspecific esterase staining.

Monocytes were purified by using a discontinuous Percoll density gradient.\textsuperscript{26} The Percoll-isolated fraction contained \(\approx 85\%\) monocytes.

Mononuclear cells and monocytes were incubated with endotoxin (*Escherichia coli* 055:B5W, Difco), captopril, indoapril (Guidotti Limited), fosinopril (Bristol-Myers Squibb), or losartan (Merck & Co) in sterile, pyrogen-free stopped test tubes at 37°C. At the end of incubation, the cells were disrupted by 3 freeze-thaw cycles and stored at \(-20°C\) until assayed.

In some experiments, freshly drawn, citrated whole blood was incubated with captopril at 37°C for 30 minutes, after which endotoxin was added, and then samples were incubated at 37°C for an additional 2 hours. Mononuclear cells were then isolated, lysed by freezing and thawing, and tested for procoagulant activity.

**Determination of TF Activity and Antigen**

Procoagulant activity was assessed by a 1-stage clotting assay.\textsuperscript{28} Results are expressed in arbitrary units (AU) by comparison with a standard curve obtained using a human brain thromboplastin standard (courtesy of Dr L. Poller, University of Manchester, Manchester, UK). This preparation was assigned a value of 1000 AU for a 100% value was assigned to the activity expressed by mononuclear cells incubated with endotoxin. Data are mean±SEM of 4 experiments. *\(P<0.05\), **\(P<0.001\).

**ACE Determination**

ACE activity was determined by the ability of the cell lysate to hydrolyze the artificial substrate H-H-L, as previously described.\textsuperscript{30} Mononuclear cells were cultured with or without endotoxin for 4 hours at 37°C, washed 3 times with HEPES-buffered saline containing 1.5 mmol/L CaCl\textsubscript{2} and 0.5 mmol/L MgCl\textsubscript{2}, resuspended at a concentration of \(5 \times 10^7\) cells/mL, and frozen until assayed. Known concentrations of histidyl-L-leucine were used to generate a standard curve.

**Statistical Analysis**

The results are given as mean±SEM. Statistical analysis was performed using the 1-way ANOVA.

**Results**

**Effect of ACE Inhibitors on Mononuclear Cell TF Activity**

Procoagulant activity was not detectable in freshly isolated mononuclear cells but was induced by exposure to 0.1 μg/mL endotoxin for 4 hours at 37°C (0.04±0.02 and 7.66±3.32 AU/\(3 \times 10^5\) mononuclear cells; \(n=4\), respectively). The procoagulant activity was characterized using either congenital factor–deficient plasmas or the monoclonal anti-TF antibody HTF1 (courtesy of Dr Yale Nemerson, Mt Sinai School of Medicine, New York, NY). The dependence on the presence of factor VII, factor X, and prothrombin and the inhibition of the activity observed with the anti-TF antibody indicates this activity to be attributable to TF (not shown).

When captopril was present during the incubation, TF activity was reduced in a concentration-dependent way, reaching \(\approx 60\%\) inhibition at a concentration of 40 μg/mL (Figure 1). The inhibition of TF activity by captopril was paralleled by a significant decrease in detectable TF antigen by ELISA analysis. Mononuclear cells expressed little TF protein on their membrane, which was dramatically increased by endotoxin (3.8±3.8 versus 859.1±246.5 pg/mL, respectively, \(n=3\)). In the presence of 20 μg/mL of captopril, TF antigen level of endotoxin-stimulated mononuclear cells was reduced to 475.6±30.3 pg/mL (\(P<0.05\)).

**Electromobility Shift Assay (EMSA)**

To determine the effect of captopril on endotoxin-induced c-Rel/p65 nuclear translocation, nuclear extracts from 4 to \(5 \times 10^7\) mononuclear cells that were endotoxin-stimulated for 1 hour in the presence or absence of captopril were prepared, and the levels of c-Rel/p65 were monitored by EMSA as previously described.\textsuperscript{29}
Because the captopril molecule contains a sulfhydryl group, which may confer properties other than ACE inhibition, such as free radical scavenging, we tested the activity of fosinopril and idrapril, 2 among the group of non–sulfhydryl-containing ACE inhibitors. Fosinopril and idrapril, incubated with mononuclear cells in the presence of endotoxin, reduced TF activity to a similar extent as captopril (Figure 2). This observation suggests that the reduction of TF observed in the presence of ACE inhibitors is attributable to inhibition of ACE.

We tested whether captopril was effective also when mononuclear cell TF was elicited in whole blood, which represents a condition closer to the in vivo situation. To this end, citrated whole blood was preincubated with captopril (10 μg/mL) for 30 minutes and then stimulated with endotoxin (0.01 μg/mL) for an additional period of 2 hours, after which mononuclear cells were isolated and disrupted. Endotoxin induced TF activity in mononuclear cells (5.84±0.87 versus 0.003±0.001 AU/3×10^6 cells, with and without endotoxin, respectively, n=3), which was significantly inhibited when 10 μg/mL captopril was present during the incubation (2.5±1.01, AU/3×10^6 cells, P<0.05).

**Effect of Losartan on Mononuclear Cell TF**

Because ACE converts angiotensin I into angiotensin II and the latter binds specific receptors, namely AT₁ and AT₂, on cell membranes,31 and because activated monocytes have been shown to express AT₁ receptors,32 we tested whether blocking the receptors could affect TF generation from endotoxin-stimulated mononuclear cells. Indeed, losartan, a specific antagonist of the AT₁ receptor family,31 downregulated endotoxin-stimulated mononuclear cell TF activity to an extent similar to that observed with captopril (Figure 3).

**Regulation of TF mRNA Levels in Endotoxin-Stimulated Mononuclear Cells by Captopril**

To determine the steady-state levels of TF mRNA, we performed reverse transcriptase–PCR. RNA prepared from untreated control cells or from cells treated with endotoxin with or without captopril was reverse transcribed and used for parallel assay of TF and GAPDH mRNA by PCR amplification. The expected 337-bp PCR product for TF was obtained. Southern blot analysis of the PCR products was performed to evaluate TF mRNA levels. No PCR product from the control cells could be detected (Figure 4). In contrast, a strong expression of TF mRNA could be observed in cells exposed to endotoxin. Captopril largely prevented the increase in TF mRNA. Southern blot analysis of GAPDH mRNA showed similar mRNA levels in control and endotoxin and captopril-treated cells, indicating that the efficiency of reverse transcription was comparable among the experimental groups. Amplification reactions performed without including reverse transcriptase gave no amplification products, thus ruling out PCR carryover.

**Effect of Captopril on Endotoxin Activation of c-Rel/p65 Heterodimers in Mononuclear Cells**

To determine whether captopril affected TF activity by preventing activation of c-Rel/p65 heterodimers, nuclear extracts of mononuclear cells exposed to endotoxin in the presence and in the absence of captopril were prepared and analyzed by EMSA. Nuclear localization of c-Rel/p65 heterodimers was induced within 1 hour after endotoxin stimulation (Figure 5). Captopril, at 20 μg/mL, almost completely abolished the translocation of c-Rel/p65 heterodimers induced by endotoxin.

To assess whether lymphocytes present in the mononuclear preparation could influence the effect of ACE inhibitors on TF, experiments were performed using preparations of Percoll-purified monocytes. Captopril consistently inhibited TF expression and mRNA levels and prevented c-Rel/p65 translocation in Percoll-purified monocytes, with an effect similar to that exerted in mononuclear cells (not shown).
of c-Rel/p65 heterodimers was determined by EMSA. (lane 3), or both (lane 4) for 30 minutes at 37°C. The presence over, losartan, a specific antagonist of the AT1 receptor family, angiotensin I and II, as well as AT1 receptors, it is conceivable that, under the appropriate conditions, monocytes generate ACE, angiotensin II is formed, and occupancy of AT1 receptor occurs. We found that endotoxin-stimulated monocytes express ACE activity in a dose-dependent fashion (data not shown), and this observation is in accordance with previous reports. Moreover, losartan, a specific antagonist of the AT1 receptor family, inhibited TF activity to an extent similar to that observed with ACE inhibitors. This result is consistent with the hypothesis that TF expression in endotoxin-activated monocytes is regulated by endogenous angiotensin II.

Induction of the TF gene in monocytes by endotoxin is regulated by transcriptional factors. Stimulation of monocytes by endotoxin induces translocation of c-Rel/p65 heterodimers from the cytoplasm into the nucleus. Binding of the heterodimers to a regulatory kB element within the TF promoter induces its transcriptional activation. It has been shown that inhibition of the nuclear translocation of c-Rel/p65 abolishes TF gene induction in human monocytes.

A role for NF-κB activity in mediating functions pertinent to atherosclerosis has recently been supported by several studies. Inflammatory or proliferative stimuli that have been established to play a role in atherogenesis activate NF-κB in several cells. Nuclear localization of the NF-κB subunit p65 can be observed in situ in smooth muscle cells, endothelial cells, and macrophages within the fibrotic intima/media and atheromatous areas of the atherosclerotic lesion. In addition, it has recently been reported that transfecting rat hearts with a cis element decoy against NF-κB binding site attenuates ischemia reperfusion injury in the myocardium. Recently, an increase in NF-κB–like activity, monocyte chemoattractant protein-1 accumulation, and neointimal macrophage infiltration has been shown in atherosclerotic vessels during accelerated atherosclerosis in rabbits. Administration of the ACE inhibitor quinapril reduced these 3 parameters, suggesting that ACE inhibitors may have a beneficial effect in early atherosclerosis. Activation of NF-κB in humans with unstable angina has been reported. Our finding that captopril largely prevents endotoxin-induced c-Rel/p65 translocation to the promoter region of the gene encoding for TF in monocytes and lymphocyte-monocyte mixed preparations provides an additional link between ACE inhibitors and the pathogenesis of atherosclerosis.

The clinical use of ACE inhibitors has been proven to decrease the incidence of recurrent myocardial infarction, suggesting for these drugs a potential antithrombotic effect. The effect of these drugs appears to be related, at least in part, to mechanisms other than their antihypertensive action. In particular, the inhibition exerted either in vitro or in vivo on the synthesis of the proinflammatory cytokines interleukin-1 and tumor necrosis factor, which are involved in the recruitment of monocytes to atherosclerotic plaques, may play a major role. Our finding that ACE inhibitors and/or angiotensin II receptor antagonists downregulate TF expression by monocytes, together with the recent observation that enalapril decreased the amount of TF antigen in patients with uncomplicated acute myocardial infarction, suggests that the antithrombotic mechanism of these drugs could be, at least in part, related to their ability in reducing TF expression in monocytes. Because monocytes/macrophages are an integral part of atherosclerotic plaques, we speculate that, in conditions in which angiotensin II biosynthesis and/or activity is suppressed, the monocyte-associated TF present in atherosclerotic plaques might also be reduced. This is of particular relevance in light of the observation that platelet deposition and thrombus formation on atherosclerotic plaques is directly related to their TF content. Thus, the described capacity of angiotensin II synthesis inhibitors and/or receptor antagonists to modulate monocyte TF expression might have important therapeutic implications in patients with cardiovascular disease.

Discussion

We provide here the first direct evidence that captopril reduces TF expression in endotoxin-stimulated human monocytes. Such an effect is attributable to ACE inhibition, given that molecules of different chemical structure, but with similar ACE inhibiting potential, exert a comparable effect. Inhibition of TF was observed at concentrations of captopril close to those reached in humans after its administration. The inhibitory action is exerted at the level of the biosynthesis of the TF protein, because captopril almost abolished the increase in mRNA levels that is observed when monocytes are exposed to endotoxin.

ACE converts angiotensin I into angiotensin II, which binds specific receptors, namely AT1 and AT2, on cell membranes. Because activated monocytes have been shown to express angiotensin I and II, as well as AT1 receptors, it is conceivable that, under the appropriate conditions, monocytes generate ACE, angiotensin II is formed, and occupancy of AT1 receptor occurs. We found that endotoxin-stimulated monocytes express ACE activity in a dose-dependent fashion (data not shown), and this observation is in accordance with previous reports. Moreover, losartan, a specific antagonist of the AT1 receptor family, inhibited TF activity to an extent similar to that observed with ACE inhibitors. This result is consistent with the hypothesis that
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