Paper

Aliskiren, a renin inhibitor, downregulates TNF-α-induced tissue factor expression in HUVECS

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Abstract

Angiotensin (Ang)II, the effector arm of the locally active renin–angiotensin system (RAS), modulates Tissue Factor (TF), the principal initiator of blood coagulation and a key promoter of atherothrombotic events. Consistent with that knowledge, previous data showed inhibitory properties of angiotensin-converting enzyme inhibitor (ACEI)s and angiotensin II type-1 receptor blocker (ARB)s, but no data are available about the effect of renin inhibition. We aimed to evaluate whether aliskiren, a direct renin inhibitor (DRI), modulates TNF-α-stimulated TF expression in cultured human umbilical vein endothelial cells (HUVECs). Zofenopril, an ACEI, and olmesartan, an ARB, were the controls. HUVECs were incubated with experimental drugs (1 nM) 30 min prior to TNF-α stimulation (0.1 ng/ml × 4 h). Main evaluation variables were procoagulant activity (single-stage clotting assay), TF antigen (ELISA) and mRNA expression (real-time polymerase chain reaction) in cell lysates. TNF-α stimulated procoagulant activity and increased TF antigen and mRNA expression. Aliskiren inhibited TNF-α-mediated TF stimulation; zofenopril and olmesartan exerted a comparable effect. We conclude that aliskiren, a DRI, downregulates TNF-α-stimulated TF expression in HUVECs, possibly as a reflection of endothelial renin activation by the cytokine.

Introduction

Tissue Factor (TF), the principal initiator of blood coagulation,[1] is the key promoter of atherothrombotic events,[2] a pathological process to which inflammation and coagulation contribute synergistically.[3] Several and interacting inflammatory stimuli modulate TF expression,[1] including Angiotensin (Ang)II[4-8] (see Celi *et al.*[9] for a review), the effector arm of locally active renin–angiotensin systems (RASs)[10] and a pro-inflammatory agonist.[11] Consistent with that concept, pharmacological angiotensin-converting enzyme (ACE) inhibition and AngII-type 1 receptor (AR) blockade downregulated TF stimulation in response to inflammatory agonists in experimental animals[12] and in vitro systems.[13,14] The RAS, however, is a multistep peptidergic system primarily controlled by renin activation, the rate-limiting step in the biological cascade to form AngII,[15] raising the issue of the effect of renin inhibition on TF expression, a so far unanswered research question.

For this reason, we evaluated the effect of aliskiren (ALI), a direct renin inhibitor (DRI),[16] on TF expression stimulated by tumor necrosis factor (TNF)-α, a well-characterised endothelial TF-stimulating pro-inflammatory cytokine[17] pathophysiologically involved in cardiovascular disease.[18] Experiments were carried out in human umbilical vein endothelial cells (HUVECs), a classical in vitro model[19] for the study of endothelial function. As a control, parallel experiments were run in the presence of either zofenopril (ZOF), an ACE inhibitor (ACEI), homogenize the form with other references or olmesartan (OLM), an AR blocker (ARB).[21]

Materials and methods

Cell isolation and culture

HUVECs were isolated from human umbilical cord veins by digestion with 0.1% collagenase (specific activity: 316 U/ml, Gibco, Invitrogen) and cultured as previously described.[22] After reaching confluence, HUVECs were detached from the flasks and plated either on gelatin-coated 96-well tissue culture plates (~30,000 cells/well) for TF procoagulant activity assay or on 6-well plates for TF enzyme-linked immunosorbent assay (ELISA) or real-time polymerase chain reaction (RT-PCR) experiments (~500,000 cells/well). HUVECs up to the fourth passage were used for all experiments. Cells were counted by light

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microscopy (six fields per replicate well) and mean cell number per experiment was estimated as the mean of three replicates.

Reagents were dissolved in saline and stored at -20°C for no longer than 2 months and diluted in serum-free RPMI at the appropriate concentrations immediately before the experiments. Upon completion, monolayer integrity and cell viability were checked by phase contrast microscopy and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay.[22]

Neither drugs nor vehicles affected cell morphology, cell number, viability, nor basal or TNF-α-stimulated TF activity and expression (data not shown). All buffers used for isolation and culture of cells were prepared with endotoxin-free water under careful pyrogen-free and sterile working conditions. Solutions were prepared in glassware rendered endotoxin-poor by heating at 180°C for 3 h.

The investigation conformed to the principles outlined in the Declaration of Helsinki for use of human tissue.

Methods

Procoagulant activity

Procoagulant activity (PCA) was assessed by a one-stage clotting time test in HUVECs disrupted by three freeze–thaw cycles as previously described.[23] In brief, disrupted cells (100 µl) were mixed with 100 µl of normal human plasma at 37°C, adding 30 s afterwards 100 µl of 25 mM $CaCl_2$ at 37°C. Time to clot formation was recorded and values converted to arbitrary units (AU) by comparison with a human brain TF calibration curve covering clotting times from 20–600 s, corresponding to 1000 and 0 AU, respectively. Incubation with an inhibitory anti-TF antibody (epitope specific for aa 1–25, American Diagnostica) or with a matched control isotype (both at 20 µg/ml) confirmed TF-dependent PCA. Experiments were run in triplicate and averaged. To restrict evaluation to appropriately responsive cell samples, only PCA values greater than 2 were used for the analysis.

TF antigen

HUVECs were washed, harvested by scraping and lysed in tris buffered saline (pH 8.5) solution containing 1% Triton X-100. Cells were disrupted by three repeated freeze–thaw cycles and debris pelleted by centrifugation at 100,000 *g* for 1 h at 4°C, and supernatants used for ELISA (Imubind TF kit, American Diagnostica). Briefly, 100 µl of the TF standards or the supernatant samples were incubated overnight at 4°C in microwells pre-coated with a murine anti-human TF capture antibody. The captured TF was detected using a biotinylated antibody fragment. Then, binding of streptavidin-conjugated horseradish peroxidase (HRP) completed the formation of the antibody–enzyme detection complex, and the addition of tetramethylbenzidine substrate and its subsequent reaction with the HRP created a blue-coloured solution. The sensitivity of the method was increased by the addition of 0.5 M sulphuric acid stop solution, which yielded a yellow colour. Solution absorbances were measured in a microplate reader at 450 nm. TF antigen levels were expressed in pg/ml using a reference curve created by the TF standards. Within- and between-assay variability was 3.5% and 5.5%, respectively.

TF real-time polymerase chain reaction

Total RNA was extracted from HUVECs using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were determined by optical density measurement via nanodrop. A mixture of 0.5 µg total RNA per sample was retrotranscribed with random primer into complementary DNA (cDNA) using the Quantitect Reverse Transcription Kit (Qiagen). The retrotranscription cycle was performed at 25°C for 5 min, 42°C for 30 min and 95°C for 3 min. RT-PCR was carried out in a Mini Opticon Real Time PCR System, and iQtm Sybr Green Supermix (Bio-Rad) was employed on the basis of the manufacturer's instructions with a final reaction of 15 µl. RT-PCR was performed under the following conditions: 95°C, 3 min; 41 cycles 95°C, 5 sec, 58°C, 20 sec. The primer sequence (Invitrogen) for RT-PCR was: TF, sense 5'-TTGGCAAGGACTTAATTTATACAC-3', antisense 5'- CTGTTCGGGAGGGAATCAC-3'; β2 microglobulin, sense 5'-CATTCCTGAAGCTGACAGCATTC-3', antisense 5'- TGCTGGATGACGTGAGTAAACC-3'; 18s, sense 5'-CTGCCCTATCAACTTTCGATGGTAG-3', antisense 5'-CCGTTTCTCAGGCTCCCTCTC-3'. All samples were analysed in triplicate and averaged. The relative expression of the target gene was normalised to the level of β2 microglobulin and 18s in the same cDNA.

Experimental design

ALI, ZOF and OLM were added at 1 nM concentration to HUVECs grown to confluence 30 min prior to TNF-α stimulation (0.1 ng/ml for 4 h) achieving about 50% of the maximal procoagulant effect, as validated in pilot experiments. Previous studies showed that the drug concentration (1 nM) used in the experimental setting provided specific and highly selective inhibition of

the pharmacological targets of ALI, ZOF and OLM, i.e. renin, ACE and AT1R, respectively.[16,20,21]

Statistics

Statistical differences were tested by two-way ANOVA. Differences among and between means were tested by the Fisher's 95% least significant difference, a procedure that limits the risk of calling each pair of means significantly different when the actual difference equals 0 to not greater than 5%.

Results

TNF-α stimulated PCA from nil to 3.38 ± 1.15 AU (n = 26, mean ± SD, $p < 0.001$), augmented antigen expression from 146 ± 22 to 799 ± 250 pg/ml (n = 6, means ± SD, $p = 0.01$), and stimulated mRNA expression by about eleven-fold (n = 4, $p < 0.001$).

ALI modulated TNF-α-stimulated PCA and down-regulated TF antigen and mRNA expression. ZOF and OLM inhibited to an extent comparable to that obtained by ALI (figures 1–3).

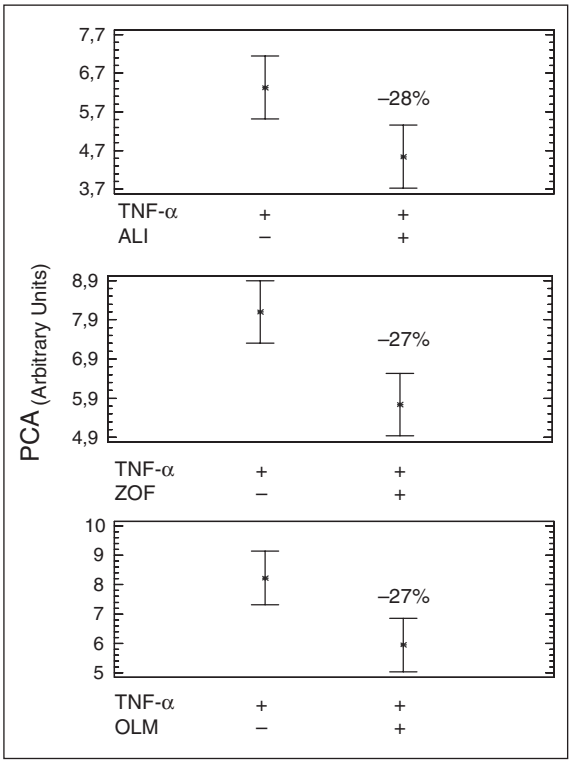

Figure 1
Effect of aliskiren (ALI, n = 7, **top panel**), zofenopril (ZOF, n = 11, **middle panel**), and olmesartan (OLM, n = 8, **bottom panel**), (all at 1 nM), on TNF-α-stimulated procoagulant activity (PCA) in HUVECs. Intervals around each mean represent a 95% least significant difference (LSD). Non-overlapping LSD intervals correspond to at least a $p < 0.05$ difference. Figure above bar reports mean percent reduction from TNF-α alone.

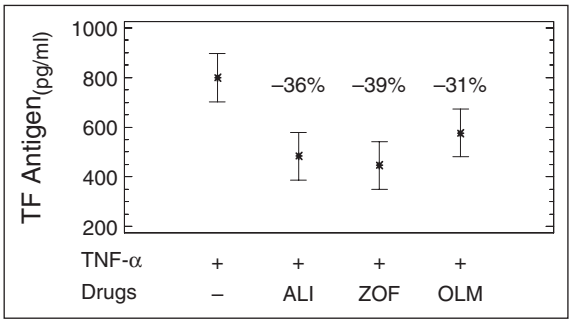

Figure 2
Effect of aliskiren (ALI), zofenopril (ZOF), and olmesartan (OLM), (all at 1 nM) on TNF-α-stimulated TF antigen expression (n = 6) in HUVECs. Intervals around each mean represent a 95% least significant difference (LSD). Non-overlapping LSD intervals correspond to at least a $p < 0.05$ difference. Figures above bars report mean percent reduction from TNF-α alone.

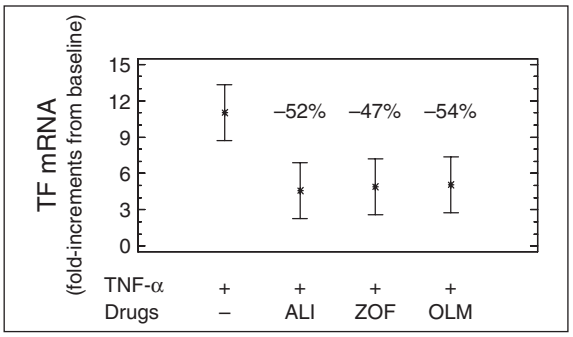

Figure 3
Effect of aliskiren (ALI), zofenopril (ZOF), and olmesartan (OLM), (all at 1 nM) on TNF-α-induced TF mRNA (RT-PCR) (n = 4). Intervals around each mean represent a 95% least significant difference (LSD). Non-overlapping LSD intervals correspond to at least a $p < 0.05$ difference. Figures above bars report mean percent reduction from TNF-α alone.

Discussion

The main and original finding of this study was to show the sensitivity to ALI, a DRI, of TF expression stimulated by TNF-α, an inflammatory cytokine involved in the pathogenesis of vascular dysfunction,[18] a piece of evidence adding a new and original facet to the understanding of the pathophysiology of abnormal endothelial states. The effect of ALI likely resulted from renin inhibition rather than pleiotropic properties of the drug, since modulation of TF activity and expression were achieved at concentrations (1 nM), well within the range shown to inhibit renin in previous studies.[16] Additional strength to our interpretation stems from the behaviour of both ZOF, an ACEI, and OLM, an ARB, that, despite the obvious chemical and pharmacological differences from ALI,[16,20,21] mitigated to a similar extent the effect of TNF-α, suggesting a common mechanism of action, i.e. inhibition of AngII-mediated effects.

Reduced TF mRNA expression in response to TNF-α stimulation in presence of ALI favours a

transcriptional site of action of the drug, a possibility that allows some plausible mechanistic inferences. In fact, TNF-α and AngII share intracellular signalling pathways including nuclear factor (NF)-kB,[18,24] a transcription factor activated by inflammatory cytokines[25] and a mandatory step for stimulation of TF mRNA by both TNF-α[26] and AngII.[12] Moreover, bidirectional cross-talk links AngII and TNF-α, by which AngII stimulates TNF-α[27] which, in turn, up-regulates all the components of the local RAS including renin,[28] angiotensinogen[29] and AngII type 1 receptors.[30] In this regard, our data expand the existing knowledge in the field by providing a first piece of evidence about the contribution of renin activation to TNF-α-induced TF stimulation in vascular endothelium. That behaviour appears to reflect a more general pattern of response, since ALI was recently shown to down-regulate adhesion molecules, NF-kB and reactive oxygen species generation in TNF-α-stimulated HUVECs.[28] Consistent with that possibility, AngII stimulated TF expression only in TNF-α pre-activated human glomerular endothelial cells,[14] while unstimulated HUVECs showed no potential to synthesise renin, angiotensinogen, AngI and AngII.[31] It is still controversial, though, whether activatable endothelial renin derives from locally synthesised stores[14,32] or exogenous sources.[33] Future studies may also help to understand the apparent contradiction by which TNF-α seems to activate renin in HUVECs and other cellular types (present data and previous studies[28-30]) while at the same time repressing its expression in renal juxtaglomerular cells.[34]

The results of our study are to be seen in the context of some limitations. First, HUVECs may not reflect endothelial cells resident in different tissues exposed to distinct regulatory factors present in the local environment that influence their ability to express TF. However, our data obtained in HUVECs agree closely with others generated in different cell lines[13,14] sharing with vascular endothelium inflammation-inducible TF synthesis in the absence of constitutive protein expression.[1] Second, we evaluated stimulated TF activity in cell lysates and not on intact cell surfaces, which reflect more accurately active TF accessible to the cellular microenvironment in vivo. Moreover, TF production in activated endothelium may not be expressed on the luminal cell surface, the site that facilitates optimal interaction with its substrates and enzyme.[35] Third, our data pertain to TNF-α, and the interaction of the cytokine with RAS may not necessarily represent a general pattern. Fourth, the molecular link(s) between TNF activation and endothelial renin production as above prospected are speculative, and need experimental verification by direct assay of renin and other components of RAS in our experimental set-up.

In conclusion, renin inhibition by ALI abrogates TNF-α-mediated stimulation of the coagulation pathway in HUVECs. The data may constitute a focus for future in vitro and in vivo studies aimed at the evaluation of the anti-thrombotic and anti-inflammatory properties of RAS blockers.

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