Cardiac Glycosides Regulate Endothelial Tissue Factor Expression in Culture

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Background—Tissue factor (TF) plays an important role in acute coronary syndromes and stent thrombosis. This study investigates whether Na\(^+/K^+\)-ATPase regulates TF expression in human endothelial cells.

Methods and Results—Ouabain inhibited tumor necrosis factor (TNF)-α–induced endothelial TF protein expression; maximal inhibition occurred at 10\(^{-3}\) mol/L, reached more than 70%, and was observed throughout the 5 hours stimulation period. The decrease in protein expression was paralleled by a reduced TF surface activity. Similarly, lowering of extracellular potassium concentration inhibited TNF-α–induced TF protein expression. In contrast, ouabain did not affect TNF-α–induced expression of full-length TF mRNA for up to 5 hours of stimulation; instead, expression of alternatively-spliced TF mRNA was upregulated after 3 and 5 hours of stimulation. Ouabain did not affect TNF-α–induced activation of the MAP kinases p38, extracellular signal-regulated kinase (ERK), and c-Jun terminal NH\(^2\) kinase; activation of Akt and p70S6 kinase remained unaltered as well. Similar to the MAP kinases, ouabain did not affect TNF-α–induced degradation of IkB-α. Ouabain had no effect on TF protein degradation.

Conclusions—Na\(^+/K^+\)-ATPase is required for protein translation of endothelial TF in culture. This observation provides novel insights into posttranscriptional regulation of TF expression. (Arterioscler Thromb Vasc Biol. 2007;27:2769-2776.)

Key Words: Ouabain • translation • thrombosis

Tissue factor (TF) is the main initiator of coagulation and thereby involved in the pathogenesis of acute coronary syndromes.\(^1\) TF antigen and activity are indeed enhanced in plaques from patients with unstable angina and myocardial infarction as compared with those from patients with stable angina.\(^2\) Moreover, increased TF plasma levels have been detected in unstable angina and acute myocardial infarction.\(^3,4\) Given its role in initiating thrombus formation, it is conceivable that TF may also be involved in the pathogenesis of stent thrombosis, which remains a major concern owing to its high morbidity and mortality.\(^5,6\) Indeed, drugs released from drug-eluting stents such as rapamycin and paclitaxel enhance endothelial TF expression and may thereby promote thrombus formation after drug-eluting stent deployment.\(^7,8\)

Na\(^+/K^+\)-ATPase is an ubiquitous transmembrane protein regulating the active transport of sodium and potassium ions across the cell membrane.\(^9\) As such, Na\(^+/K^+\)-ATPase is important for cellular homeostasis. It is not known, however, whether Na\(^+/K^+\)-ATPase influences TF expression and thereby possibly thrombus formation. Cardiac glycosides such as ouabain and digoxin inhibit Na\(^+/K^+\)-ATPase activity in a very specific manner.\(^10,11\) As these drugs impair vascular smooth muscle cell (VSMC) proliferation at higher concentrations,\(^12\) a concomitant negative effect on endothelial TF expression would render them interesting candidate drugs for application on drug-eluting stents. This study was therefore designed to examine the effect of cardiac glycosides on endothelial TF expression.

Materials and Methods

Cell Culture

Human aortic endothelial cells (HAECs) were obtained from Clonetics and cultured as described.\(^13,14\) Cells were grown to confluence in 3-cm culture dishes, rendered quiescent for 24 hours in medium containing 0.5% FCS, and stimulated with 5 ng/mL TNF alpha (TNF-α; R&D Systems). Ouabain, digoxin, carbenoxolone, glycyrrhizic acid, and cycloheximide (all from Sigma) were added to the dishes 30 minutes before stimulation. In some experiments, the medium was replaced 30 minutes before stimulation with Dulbecco’s Modified Eagle Medium (DMEM) containing reduced potassium concentrations and sodium concentrations adjusted to maintain osmolarity. Cytotoxicity was assessed by a colorimetric assay for...
detection of lactate dehydrogenase (LDH) according to the manufacturer’s recommendations (Roche).

### Western Blot Analysis

Western blot analysis was performed as described. Cells were lysed in 50 mmol/L TRIS buffer, 25 μg protein were loaded per lane, and 10% SDS-PAGE was performed. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) by semidy. An antibody against human TF (American Diagnostica) was used at 1:2000 dilution; antibodies against vascular cell adhesion molecule (VCAM)-1 (R&D Systems) and heme oxygenase-1 (HO-1; Stressgen) were used at 1:1’000 and 1:5’000 dilution, respectively. An antibody against α-tubulin (aT, 1:20’000, Sigma) was applied as loading control. Antibodies against phosphorylated p38 MAP kinase (p38), phosphorylated p44/42 MAP kinase (ERK), and phosphorylated c-Jun terminal NH2 kinase (JNK) (all from Cell Signaling) were used at 1:1’000, 1:5’000, and 1:1’000 dilution, respectively. Antibodies against total Akt, phosphorylated Akt (Akt), phosphorylated p70S6 kinase (p70S6K), total Akt, and total p70S6K (all from Cell Signaling) were used at 1:2’000, 1:5’000, and 1:1’000 dilution, respectively. Antibodies against phosphorylated Akt (Akt), phosphorylated p70S6 kinase (p70S6K), total Akt, and total p70S6K (all from Cell Signaling) were applied at 1:1’000, 1:2’000, 1:1’000, and 1:2’000 dilution, respectively. Antibody against c-Jun (SantaCruz) was used at 1:2’500 dilution. Proteins were detected with a horseradish peroxidase linked secondary antibody (Amersham).

### Real-Time PCR

Total RNA was extracted from HAECs with 1 mL TRIzol Reagent (Invitrogen) according to the manufacturer’s recommendations. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Bioscience) in a final volume of 33 μL using 4 μg of RNA. The total cDNA pool obtained served as a template for subsequent PCR amplification with primers specific for full-length TF (sense primer: 5’-TCCCCCAGATGCACCTTACCC-3’; bases 508 to 529 of F3 cDNA; NCBI no. NM 001993; antisense primer: 5’-CCCTTCTCTGCAGCCCATACAC-3’; bases 843 to 863 of F3 cDNA; NCBI no. NM 001993), or primers not discriminating full-length and alternatively-spliced TF (sense primer: 5’-TCCCCCAGATGCACCTTACCC-3’; bases 508 to 529 of F3 cDNA; NCBI no. NM 001993; antisense primer: 5’-TGACCACAAATACCACAGCTCC-3’; bases 892 to 913 of F3 cDNA; NCBI no. NM 001993). Real-time PCR amplification was performed in a Mx3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma) in 25 μL final reaction volume containing 2 μL cDNA, 10 pmol of each primer, 0.25 μL of internal reference dye, and 12.5 μL of JumpStart Taq ReadyMix (buffer, dNTP, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). The following cycling parameters were used: 95°C for 10 minutes in cycle, 95°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute, for a total of 40 cycles. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. L28 primers served as loading control. Products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. The identity of alternatively spliced TF PCR products was confirmed by extraction from the agarose gel and sequencing (Microsynth AG).

### TF Surface Activity

TF surface activity was analyzed with a colorimetric assay (American Diagnostica) according to the manufacturer’s recommendations with some modifications as described. Cells were grown to confluence in 12-well plates. After stimulation with TNF-α, cells were washed twice with phosphate-buffered saline and incubated with human FVIIa and FX at 37°C, allowing for the formation of the TF/FVIIa complex at the cell surface. This complex converted human FX to FXa, which was measured by its ability to cleave a chromogenic substrate. A standard curve was established with lipitated human TF to assure that the results were in the linear range of detection (data not shown).

### ELISA

Expression of tissue-type plasminogen activator-1 (tPA) was analyzed with an ELISA assay (American Diagnostica) according to the manufacturer’s recommendations. Cells were grown to confluence in 6-well plates and stimulated with TNF-α with or without ouabain pretreatment (10^{-5} mol/L, 50 μL of the media was used for ELISA. To detect tPA in a linear range of detection, a standard curve was established with provided tPA standards (data not shown).

### Na+/K+-ATPase Activity

Unidirectional K⁺-influx into HAECs was measured using ⁸⁶Rb as a radioactive tracer. HAECs were pretreated with ouabain at 10^{-6} to 10^{-3} mol/L for 15 minutes before addition of ⁸⁶Rb. To estimate unidirectional K⁺-uptake, ⁸⁶Rb accumulation in cells was monitored in control and in ouabain-treated cells. Radioactivity of cell lysates as well as that of the aliquots of incubation medium was measured using Packard Tri-Carb 1600 TR liquid scintillation counter (Packard, Meriden, CT). Unidirectional K⁺-influx was calculated from the slope in the linear part of the ⁸⁶Rb accumulation curve plotted against incubation time and normalized to protein content in cell lysates.

### Statistical Analysis

Data are given as mean±SEM. Unpaired Student t test was performed for statistical analysis. A probability value <0.05 denoted a significant difference.

### Results

**Ouabain Inhibits TF Protein Expression and Surface Activity**

HAECs were stimulated with 5 ng/mL TNF-α for 5 hours in the presence or absence of ouabain (10^{-8} to 10^{-5} mol/L). TNF-α induced a 14-fold increase in TF protein expression as compared with baseline (n=10; P<0.0001; Figure 1A and 1B). Ouabain inhibited TNF-α–induced TF expression in a concentration-dependent manner (n=5; P<0.001; Figure 1A); a maximal effect was observed at a concentration of 10^{-5} mol/L and reached more than 70%. Similar to ouabain, digoxin decreased TNF-α–induced TF expression in a concentration-dependent manner (n=5; P<0.05; Figure 1D). Ouabain blunted TNF-α–induced TF expression already after 1 hour of stimulation, and this effect was maintained throughout the stimulation period (n=4; P<0.05; Figure 1B). Consistent with these observations, ouabain impaired TNF-α–induced TF surface activity by 44% (n=5; P<0.0001; Figure 1C). Neither ouabain nor digoxin affected basal TF expression (n=5; P=NS; Figure 1A and 1D).

Similar to its effect on TF expression, ouabain inhibited TNF-α–induced VCAM-1 protein expression; a 97% decrease was observed at 10^{-7} mol/L (n=5; P<0.0001; Figure 1E). In contrast, expression of HO-1 and αT was not affected (n=4 for HO-1, and n=5 for αT, respectively; P=NS; Figure 1E). Moreover, expression of tPA was not affected measured by ELISA (n=5; P=NS; Figure 1F).

Ouabain has been described to interfere with the function of gap junctions. To investigate whether such an action may be involved in its effect on TF expression, cells were treated with carbexoxol, a well-known gap junc-
was observed in potassium-free medium, where TNF-α stimulation in a concentration-dependent manner; a maximal effect of ouabain (10^{-5} mol/L) did not affect LDH release at all the concentrations used (n=8; P=NS; data not shown).

Ouabain, digoxin, carbenoxolone, and glycyrhrizic acid did not affect LDH release at all the concentrations used (n=5; P=NS; data not shown). Ouabain and digoxin slightly altered endothelial cell morphology during incubation; the cells appeared rounded, but remained attached to the dishes. The effect on TF expression preceded these morphological changes, which became only evident after more than 2 hours (n=5; data not shown).

**Lowering Extracellular Potassium Concentrations Decreases TF Protein Expression**

Extracellular potassium concentration was lowered to impair Na^{+}/K^{+}-ATPase activity.22 Decreasing the extracellular potassium concentration inhibited TNF-α induced TF expression in a concentration-dependent manner; a maximal effect was observed in potassium-free medium, where TNF-α-induced TF expression was reduced by 77% (n=4; P<0.0001; Figure 2A).

**Ouabain Inhibits Na^{+}/K^{+}-ATPase Activity**

Unidirectional K^{+}-influx into HAECs was measured using ^{86}Rb as a radioactive tracer. Active Na^{+}/K^{+}-ATPase-mediated K^{+}-influx comprised 86% of total K^{+}-influx into HAECs. Ouabain completely inhibited Na^{+}/K^{+}-ATPase activity at a concentration of 10^{-4} mol/L, whereas it decreased Na^{+}/K^{+}-ATPase activity by 43% and 61% at 10^{-6} and 10^{-5} mol/L, respectively (n=5; P<0.05; Figure 2B).

**Ouabain Does Not Affect Expression of Full-Length TF mRNA, but Upregulates Alternatively-Spliced TF mRNA**

Real-time PCR revealed that TNF-α (5 ng/mL) induced expression of TF mRNA with a maximal effect occurring after 2 hours. Induction of full-length TF mRNA was not affected by ouabain (10^{-5} mol/L) (n=5; P=NS for each time point; Figure 3A). In contrast, when TF mRNA expression was assessed with primers not discriminating full-length and alternatively-spliced TF, treatment with ouabain (10^{-5} mol/L) did not affect TF mRNA expression for up to 2 hours, whereas expression was enhanced by 2.0-fold after 3 hours (n=5; P<0.05; Figure 3B), and 3.3-fold after 5 hours (n=5; P<0.01; Figure 3B). Agarose gel electrophoresis of the amplification products revealed a band at 384 bp, present at all time points examined and corresponding to full-length TF, as well as a second band at 224 bp appearing only after 3 and 5 hours of stimulation (Figure 3B); the PCR product corresponding to this band was sequenced and identified as alternatively-spliced TF. Hence, the ouabain-induced increase in total TF mRNA expression at 3 and 5 hours occurred because of upregulation of alternatively-spliced TF mRNA.
Ouabain Does Not Affect Activation of MAP Kinases

TNF-α induced a transient phosphorylation of the MAP kinases p38, ERK, and JNK, with maximal activation occurring after 15 minutes of stimulation.\textsuperscript{23,24} The activation pattern of these MAP kinases was not affected by ouabain (10^{-5} mol/L) at all time points examined (n=4; P=NS for each time point; Figure 4A). Similarly, activation of Akt and p70S6K remained unaffected by ouabain (n=4; P=NS for each time point; Figure 4B). Neither TNF-α nor ouabain altered total expression of MAP kinases, Akt, or p70S6K.

Figure 2. Lowering extracellular potassium concentration decreases TNF-α-induced TF protein expression. A, Lowering extracellular potassium concentration decreases TNF-α-induced TF protein. B, Ouabain inhibits Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity assessed by \textsuperscript{86}Rb-influx.

Figure 3. Ouabain does not affect expression of full-length TF mRNA, but enhances alternatively-spliced TF mRNA. A, Ouabain does not affect TNF-α-induced full-length TF mRNA expression. B, Ouabain enhances TNF-α-induced total TF mRNA expression at 3 and 5 hours.
Figure 4. Ouabain does not affect p38, ERK, JNK, Akt, p70S6K, and NFκB activation. TNF-α-induced phosphorylation of p38, ERK, JNK (A) as Akt and p70S6K (B) is not affected by ouabain. C, TNF-α-induced degradation of IκB-α is not affected by ouabain.
Degradation of IкB-α was not altered by ouabain as compared with control (n=5; P=NS for each time point; Figure 4C).

**Ouabain Does Not Affect TF Protein Degradation**

HAECS were first pretreated with cycloheximide, an inhibitor of protein translation, to block de novo protein synthesis before stimulation with TNF-α (5 ng/mL) for 3 hours.25 Cycloheximide (5 μg/mL) abolished TNF-α induced TF protein expression (n=3; P=0.0001; data not shown) without exerting any toxicity as determined by LDH release (n=7; P=NS; data not shown). To assess whether ouabain promotes TF protein degradation, TF protein expression was induced by TNF-α (5 ng/mL) for 3 hours and observed to remain stable from 3 to 7 hours after TNF-α stimulation (n=5; P=NS for each time point versus TNF-α stimulation at 3 hours; Figure 5A); then, ouabain (10−7 mol/L) was added in the presence or absence of cycloheximide (5 μg/mL) after 3 hours of TNF-α stimulation. Ouabain alone did not affect the level of TF protein expression throughout this time window (n=5, P=NS for both TF protein expression and trend-line of TNF-α plus ouabain versus TNF-α alone; Figure 5B). Cycloheximide alone slightly reduced TF protein level from 3 to 7 hours after TNF-α stimulation (n=4; P=NS for both TF protein expression and trend-line of TNF-α plus cycloheximide versus TNF-α alone; Figure 5C). Ouabain did not affect TF protein level in the presence of cycloheximide (n=4; P=NS for both TF protein expression and trendline of TNF-α plus cycloheximide plus ouabain versus TNF-α plus cycloheximide; Figure 5D).

**Discussion**

This study demonstrates that cardiac glycosides such as ouabain and digoxin inhibit TNF-α-induced endothelial TF protein expression and surface activity. Cardiac glycosides specifically inhibit Na+/K+-ATPase activity by binding to its α subunit.9–11 Ouabain indeed inhibited Na+/K+-ATPase activity in HAECs as previously described in human arterial and venous endothelial cells.11,26,27 Consistently, inhibition of Na+/K+-ATPase, via reduced extracellular potassium concentrations, blunted TNF-α-induced endothelial TF expression. Ouabain has been described to inhibit gap junctions as well18,28; however, an involvement of gap junctions in regulating TF expression can be ruled out, because the gap junction inhibitor carbenoxolone did not affect endothelial TF protein expression. Hence, Na+/K+-ATPase activity is required for induction of endothelial TF protein expression in culture.

Slight alterations in endothelial cell morphology after cardiac glycoside treatment have been described previously and been attributed to an effect of Na+/K+-ATPase on adhesion molecules.22,29,30 The effect on TF expression preceded these morphological changes. A nonspecific action of cardiac glycosides on cell viability can be excluded, as LDH release was not enhanced; moreover, ouabain (10−7 mol/L) is known to exert an antiapoptotic effect on human umbilical vein endothelial cells,31 and to reduce VSMC death during up to 30 hours of treatment (10−3 mol/L).32

TNF-α induces TF expression via activation of the MAP kinases p38, ERK, and JNK.15 TNF-α-induced phosphorylation of these MAP kinases was not affected by ouabain. Consistently, ouabain did not alter TNF-α–induced expression of full-length TF mRNA, indicating that the effect of ouabain on TF expression occurs at the posttranscriptional level. Furthermore, ouabain did not enhance TF protein degradation, implying that ouabain inhibits TF protein translation. Such an effect is consistent with a limited transfer of amino acids from aminoacyl soluble ribonucleic acid to polypeptide in the presence of low intracellular potassium concentrations, which has been documented in different cell lines.33–35 Hence, decreased intracellular potassium concentrations seem to determine translational regulation of endothelial TF expression, probably at the level of polypeptide formation. Posttranscriptional regulation of TF has not been
studied as well as transcriptional mechanisms; indeed, translational regulation of endothelial TF has only rarely been observed before.\textsuperscript{7}

In contrast to full-length TF mRNA, TNF-\(\alpha\)-induced expression of total TF mRNA was enhanced after 3 and 5 hours of ouabain treatment. At these time points, a second transcript appeared as a smaller-sized band on the agarose gel. This band was sequenced and identified as alternatively-spliced TF; thus, the ouabain induced increase in total TF mRNA expression occurring after 3 and 5 hours was attributable to an increase in expression of alternatively-spliced TF mRNA. Alternatively-spliced TF is expressed and released from cytokine stimulated endothelial cells.\textsuperscript{16,36} The increase in expression of alternatively-spliced TF mRNA in response to ouabain, however, probably lacks any biological significance, because ouabain concomitantly inhibits TF translation. Despite this observation, an increase in alternatively-spliced TF protein cannot be ruled out completely.

Endogenous ouabain has been isolated from human plasma\textsuperscript{37,38}; its biological role still remains controversial. In humans, conditions such as physical exercise, essential hypertension, and congestive heart failure are associated with increased plasma concentrations of endogenous ouabain.\textsuperscript{39} In human plasma, concentrations of 10\textsuperscript{10}–10\textsuperscript{11} mol/L have been measured\textsuperscript{37,40}; in view of such concentrations, endogenous ouabain does not seem to regulate TF expression. The reference range for serum concentrations of cardiac glycosides such as digoxin is around 10\textsuperscript{10}–10\textsuperscript{11} mol/L\textsuperscript{41,42}; moreover, a narrow therapeutic range limits systemic application of cardiac glycosides at higher concentrations. Therefore, systemically applied glycosides do not seem to alter TF expression. However, cardiac glycosides may be applied locally at much higher concentrations, for example on drug-eluting stents. Stent thrombosis remains a major concern after drug-eluting stent deployment because of its high morbidity and mortality.\textsuperscript{5,6,43} TF may be induced by stenting and is a key factor in the pathogenesis of stent thrombosis; both antiproliferative agents used on drug-eluting stents, paclitaxel and rapamycin, do indeed enhance endothelial TF expression and thereby promote thrombus formation.\textsuperscript{7,8} Similar to rapamycin and paclitaxel, cardiac glycosides exerts antiproliferative effects on human vascular smooth muscle cells,\textsuperscript{12} but in contrast suppresses endothelial TF expression and thereby may reduce late stent thrombosis. Thus, cardiac glycosides may represent an interesting candidate drug to be applied on drug-eluting stents. In conclusion, this study indicates that Na\(^{+}/K\(^{+}\))-ATPase regulates endothelial TF expression at the level of protein translation. This observation provides novel insights into posttranscriptional regulation of endothelial TF expression and opens new clinical applications of cardiac glycosides.

Sources of Funding

This work was supported by the Swiss National Science Foundation (grant No. 3200B0-113328/1 to F.C.T. and grant No. 3100-068118/02/1 to T.F.L.), the Bonizzi-Theler Foundation, the Velux Foundation, the Wolffermann Nägeli Foundation, and the Swiss Heart Foundation.

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2007;27:2769-2776
doi: 10.1161/ATVBAHA.107.153502
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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