Amiodarone Inhibits Arterial Thrombus Formation and Tissue Factor Translation

A. Breitenstein, S.F. Stämpfli, G.G. Camici, A. Akhmedov, H.R. Ha, F. Follath, A. Bogdanova, T.F. Lüscher, F.C. Tanner

Background—In patients with coronary artery disease and reduced ejection fraction, amiodarone reduces mortality by decreasing sudden death. Because the latter may be triggered by coronary artery thrombosis as much as ventricular arrhythmias, amiodarone might interfere with tissue factor (TF) expression and thrombus formation.

Methods and Results—Clinically relevant plasma concentrations of amiodarone reduced TF activity and impaired carotid artery thrombus formation in a mouse photochemical injury model in vivo. PTT, aPTT, and tail bleeding time were not affected; platelet number was slightly decreased. In human endothelial and vascular smooth muscle cells, amiodarone inhibited tumor necrosis factor (TNF)-α and thrombin-induced TF expression as well as surface activity. Amiodarone lacking iodine and the main metabolite of amiodarone, N-monodesethylamiodarone, inhibited TF expression. Amiodarone did not affect mitogen-activated protein kinase activation, TF mRNA expression, and TF protein degradation. Metabolic labeling confirmed that amiodarone inhibited TF protein translation.

Conclusions—Amiodarone impairs thrombus formation in vivo; in line with this, it inhibits TF protein expression and surface activity in human vascular cells. These pleiotropic actions occur within the range of amiodarone concentrations measured in patients, and thus may account at least in part for its beneficial effects in patients with coronary artery disease. (Arterioscler Thromb Vasc Biol. 2008;28:2231-2238.)

Key Words: amiodarone ■ tissue factor ■ thrombosis ■ SCD

Sudden cardiac death (SCD) is defined as an unexpected fatal event occurring within 1 hour after the onset of symptoms in an apparently healthy subject or patients with an underlying cardiac disease not severe enough to predict such a fatal outcome.1 About 400,000 SCD occur every year in the United States,2 more than 75% in patients with coronary artery disease.3-5 The most common pattern leading to SCD is acute arterial thrombus formation initiating fatal ventricular arrhythmias6; indeed, in several postmortem studies, acute thrombosis was identified in the majority of coronary arteries examined.6-8

Tissue factor (TF), as the main initiator of the coagulation cascade, is essential for arterial thrombus formation.9 TF expression in endothelial cells is induced by different inflammatory mediators including tumor necrosis factor (TNF)-α,10 interleukin (IL)-1β,11 or histamine,12 and, consistent with these observations, TF expression is upregulated in atherosclerotic plaques of patients with unstable angina and myocardial infarction.13

Amiodarone [2-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran] is widely used for the treatment of atrial and ventricular arrhythmia,14-16 even though it was originally introduced as a potent coronary vasodilator.17,18 Belonging to class III antiarrhythmic drugs,19 amiodarone is a potassium channel inhibitor in cardiac myocytes and is known, despite its clinical effectiveness, for its low proarrhythmic potential. In a large meta-analysis of 13 randomized trials of prophylactic amiodarone treatment in patients with symptomatic compensated congestive heart failure (ejection fraction ≤40%) or after acute myocardial infarction (with variable ejection fraction), amiodarone reduced overall rates of SCD by 29% resulting in a 13% reduction in total mortality.20 To date it is not known whether the beneficial effects attributed to amiodarone are solely attributable to its electrophysiological properties or whether other mechanisms, such as inhibition of arterial thrombus formation, might be involved as well. This study was therefore designed to investigate the effect of amiodarone on thrombus formation and TF, the key initiator of the coagulation cascade.
Materials and Methods

For the detailed Materials and Methods please see the supplemental Materials and Methods (available online at http://atvb.ahajournals.org). Briefly, C57Bl/6 mice were treated with amiodarone (80 mg/kg with an intraperitoneal injection every 24 hours for 7 days) or vehicle (1.43% Tween 80, 0.29% benzyl alcohol), respectively. The photochemical injury model was performed 24 hours after the last application as described previously.21 The concentration of amiodarone and MDEA was measured in mouse plasma and aorta by high-performance liquid chromatography (HPLC). PTT, aPTT, tail bleeding time, and platelet number were determined to investigate systemic coagulation parameters.

Human aortic endothelial cells (HAECs; Clonetics, Allschwil, Switzerland) and human aortic vascular smooth muscle cells (HAVSMCs; Clonetics) were cultured as described.22 Protein expression was determined by Western blot analysis, and RNA by real-time polymerase chain reaction (PCR) after isolation with TRIzol Reagent (Invitrogen). Further, TF activity was measured with a colorimetric assay (American Diagnostica).

Results

Amiodarone Inhibits TF Activity and Arterial Thrombosis In Vivo at Clinically Relevant Plasma and Tissue Concentrations

Mice were treated with amiodarone (80 mg/kg body weight, intraperitoneal injection) or vehicle every 24 hours for 7 days. Amiodarone and MDEA concentrations were determined in mouse plasma as well as in thoracic aorta. Amiodarone plasma concentration was 1.53±0.17 μmol/L (1.05±0.12 mg/L), and tissue levels reached a concentration of 64.08±7.59 μmol/g (43.69±5.18 μg/g) (n=4; Figure 1A and 1B). MDEA levels were lower in both plasma (0.34±0.08 μmol/L [0.24±0.05 mg/L]) and tissue (3.53±0.05 μmol/g [2.31±0.33 μg/g] n=4; Figure 1A and 1B). Vehicle-treated mice developed carotid artery thrombosis within a mean occlusion time of 39.6±3.0 minutes, whereas amiodarone-treated mice occluded within a mean time period of 83.3±12.1 minute (n=7; P<0.005; Figure 1C). Initial blood flow in carotid artery did not differ between vehicle- and amiodarone-treated mice (0.72±0.06 versus 0.85±0.01 mL/min; n=7; P=NS). In contrast to the 7-day treatment, a single bolus treatment with amiodarone did not affect arterial thrombus formation (31.4±2.7 minutes [control] versus 25.1±1.3 minutes [amiodarone]; n=5; P=NS). The 7-day amiodarone treatment impaired TF activity in mouse carotid artery in vivo (n=5; P<0.05; Figure 1D). PTT and aPTT remained unaltered (PTT: 11.50±0.11 seconds [control] versus 11.19±0.15 seconds [amiodarone]; P=NS; n=7; supplemental Table I) aPTT: 20.24±0.79 seconds [control] versus 20.70±0.83 seconds [amiodarone]; P=NS; n=7; supplemental Table I). Total platelet number was decreased by 25% (1056±22×10³/μL [control] versus 789±42×10³/μL [amiodarone]; P<0.05; n=7; Table I), while total leukocyte number was not affected (3.42±0.06 versus 3.03±0.11; P=NS; supplemental Table I). Tail bleeding time remained unchanged (393.0±145.0 seconds [control] versus 349.3±92.3 seconds [amiodarone]; n=7; P=NS; supplemental Table I).

Amiodarone Inhibits TF Protein Expression and Surface Activity

HAECs were stimulated with TNF-α (5 ng/mL) or thrombin (1 U/mL) for 5 hours in the presence or absence of amiodarone. Amiodarone inhibited TNF-α and thrombin-induced TF protein expression in a concentration-dependent manner; a significant effect was observed at 3 μmol/L, whereas the maximal effect occurred at 10 μmol/L and reached more than 80% inhibition (TNF-α: n=4; P<0.001; Figure 2A; thrombin: n=4; P<0.005; Figure 2B). Amiodarone blunted TNF-α–induced TF protein expression throughout the time course from 1 to 5 hours after stimulation (n=4; P<0.01; Figure 2C), and, consistent with its effect on TF expression, impaired TF surface activity (n=4; P<0.02; Figure 2D). Amiodarone also inhibited TF protein expression in HAVSMCs (n=6; P<0.005; Figure 2E). In contrast, amiodarone did not induce any changes in cell morphology, and no signs of toxicity were detected by LDH release for all concentrations applied (n=4; P=NS; data not shown).

MDEA and AMWI Inhibit TF Protein Expression

MDEA, the major metabolite of amiodarone in vivo,3 inhibited TF protein expression in TNF-α–stimulated HAECs at 3 μmol/L (n=4; P<0.02; Figure 3A), whereas it was toxic at higher concentrations as determined by LDH release (n=4; P<0.001; data not shown). AMWI suppressed TF protein expression to a similar extent as amiodarone (n=5; P<0.05; Figure 3B). No cell damage was detected by LDH release for all concentrations of AMWI (n=5; P=NS; data not shown).
Figure 2. Amiodarone inhibits TF protein expression and surface activity. A, Amiodarone inhibits TNF-α–induced TF protein expression in human endothelial cells (*P<0.001 vs TNF-α alone). B, Amiodarone inhibits thrombin-induced TF protein expression (*P<0.05 vs thrombin alone). C, Amiodarone inhibits TNF-α–induced TF protein expression throughout the time course of stimulation (*P<0.01 vs TNF-α alone for 3, 4, and 5 hours). D, Amiodarone inhibits TNF-α–induced TF surface activity (*P<0.02 vs TNF-α alone). E, Amiodarone inhibits TNF-α–induced TF protein expression in human vascular smooth muscle cells (*P<0.005 vs TNF-α alone).
Amiodarone Does Not Affect TFPI
Neither basal nor TNF-α-induced TFPI protein expression was affected by amiodarone (n=4; P=NS; Figure 3C).

Amiodarone Does Not Affect TF mRNA Expression
Real-time PCR revealed that TNF-α (5 ng/mL) induced TF mRNA expression within 1 hour (n=5; P<0.01; Figure 4A). Amiodarone (10 μmol/L) did not affect TF mRNA expression (n=5; P=NS for each time point; Figure 4A).

Amiodarone Does Not Affect Activation of MAP Kinases and NFκB
TNF-α (5 ng/mL) induced a transient phosphorylation of the MAP kinases p38, extracellular signal regulated kinase
Cycloheximide (5 ng/mL), amiodarone (10 μmol/L) together with cycloheximide do not promote TF protein degradation (10^4/H9262 H9251 H9240 H11005 H11004 H9240 H9251 H11006 H11005 H11021 H11005 H11006 H11005 H11006 P P NS versus each time point; Figure 5B). Cycloheximide alone did not alter TF protein degradation (n=4; P=NS versus TF-α for each time point; Figure 5B). Similarly, amiodarone did not affect TF protein degradation, neither alone (n=4; P=NS versus TF-α for each time point; Figure 5B) nor together with cycloheximide (n=4; P=NS versus TF-α for each time point; Figure 5B).

Amiodarone Does Not Promote TF Protein Degradation
HAECS were stimulated with TNF-α (5 ng/mL) for 3 hours before addition of cycloheximide (5 ng/mL), amiodarone (10 μmol/L), or both. TF protein expression remained stable under control conditions from 3 to 7 hours after stimulation (n=4; P=NS versus TF-α for each time point; Figure 5B). Cycloheximide alone did not alter TF protein degradation (n=4; P=NS versus TF-α for each time point; Figure 5B). Similarly, amiodarone did not affect TF protein degradation, neither alone (n=4; P=NS versus TF-α for each time point; Figure 5B) nor together with cycloheximide (n=4; P=NS versus TF-α for each time point; Figure 5B).

Discussion
This study shows that amiodarone, at clinically relevant concentrations, impairs thrombus formation, arterial TF activity, and total platelet number in vivo. Further, it demonstrates that amiodarone reduces TF surface activity and protein expression in human vascular cells by an inhibition of TF translation. These results reveal a novel antithrombotic pleiotropic action of amiodarone.

Patients receiving long-term amiodarone treatment (200 mg orally per day) exhibit plasma concentrations of amiodarone and MDEA averaging 1.70±0.63 μmol/L (1.16±0.43 mg/L) and 1.48±0.53 μmol/L (0.97±0.35 mg/L), respectively.23,26 Higher levels are observed with a dose of 600 mg per day averaging 5.08±2.20 μmol/L (3.46±1.5 mg/L) for amiodarone and 4.26±1.78 μmol/L (2.79±1.17 mg/L) for MDEA.26,27 After intravenous application of amiodarone, concentrations as high as 11.74 μmol/L (8 mg/L) are observed.28 Because of their lipophilic properties, both amiodarone and MDEA accumulate to high concentrations in various tissues;26,27,29 indeed, in human myocardium, amiodarone and MDEA levels reach 12.3 to 22.9 μmol/g (8.4 to 15.6 μg/g), and 42.3 to 78.0 μmol/g (27.7 to 51.1 μg/g), respectively, and 10 times higher concentrations were measured in epicardial fat tissue (153.75 μmol/g [105.0 μg/g]).27 Mice for the in vivo experiments were treated with amiodarone for 7 days to reach steady-state plasma and tissue levels of the drug. Mouse amiodarone plasma concentrations

Amiodarone Inhibits TF Protein Translation
The effect of amiodarone on TF protein translation was assessed by a metabolic pulse labeling experiment using [35S]-methionine/cysteine as radioactive tracer. Amiodarone (10 μmol/L) impaired TF protein synthesis as demonstrated by a diminished incorporation of methionine/cysteine into the immunoprecipitated protein (n=4; P<0.01 versus TNF-α alone; Figure 5A). Unspecific binding of the immunoprecipitated antihuman TF antibody could be excluded using nonimmune IgG (Figure 5A).

Because potassium is essential for protein synthesis23 and ouabain, a known Na+/K+-ATPase inhibitor, impairs TF protein translation in HAECS by lowering the intracellular potassium concentration,25 amiodarone may influence TF expression by a similar mechanism. Intracellular K⁺ concentration under control conditions was 0.7±0.07 μmol/mg protein (n=4). Ouabain (1 μmol/L) decreased the K⁺ concentration by 60% (0.27±0.03 μmol/mg; n=4; P<0.005 versus control), while amiodarone (10 μmol/L) did not affect intracellular K⁺-concentration (0.7±0.05 μmol/mg protein; n=4; P=NS).
(1.53±0.17 μmol/L [1.05±0.12 μg/mL]) as well as in the aortic vessel wall (64.08±7.59 μmol/g [43.69±5.18 μg/g]) equal plasma and tissue levels in humans under chronic amiodarone therapy. Nevertheless, mouse plasma and tissue concentrations of MDEA were lower than those of amiodarone, which is in contrast to findings in humans, where comparable or even higher MDEA levels are observed.23,26 This observation is in line with previous studies and may be attributable to a different liver metabolism in the two species.30,31 Further, the amiodarone and MDEA concentrations applied in our in vitro study (1 to 10 μmol/L) are within a clinically relevant range. Hence, the herein observed antithrombotic action of amiodarone may well represent an additional mechanism by which amiodarone reduces SCD.

TF is the key initiator of coagulation and therefore is an essential trigger for thrombosis.32,33 In fact, reducing TF expression impairs thrombus formation in vivo, thus underscoring this concept.9 Plaque rupture, a frequent initial event in acute coronary syndromes, leads to the exposure of TF to the circulation resulting in acute thrombus formation. In addition, prothrombin is expressed by vascular smooth muscle cells34 and exposed to the circulating blood under these conditions; once activated in response to TF-induced coagulation this vessel wall–derived prothrombin may well contribute to thrombus formation, underlying the central role of TF. To investigate arterial thrombosis in vivo, a photochemical vascular injury model was used as it is an established method to study TF-dependent thrombus formation.9 Mean occlusion time in amiodarone-treated mice was more than doubled, indicating that clinically relevant concentrations of amiodarone do indeed inhibit arterial thrombus formation in vivo. The reduced TF activity in mouse carotid artery indicates that amiodarone negatively regulates thrombus formation at least in part by inhibiting TF in vivo. Amiodarone is known to be a potent vasodilator in veins and arteries. A contribution of such an acute effect to the increase in mean occlusion time can be excluded, because arterial thrombus formation after a bolus treatment with amiodarone did not reveal any difference as compared to the control group. Moreover, an involvement of the extrinsic and intrinsic pathway of the coagulation cascade could be ruled out as PTT and aPTT were not affected by amiodarone. Nevertheless, the reduced total platelet count suggests that platelets may contribute to the amiodarone-mediated antithrombotic effect.

To assess the relevance of the amiodarone effect in human cells, we treated human primary vascular cells with clinically relevant amiodarone concentrations. Indeed, amiodarone exhibited a consistent effect on TF activity as well as protein expression; furthermore, the unaltered expression of TFPI excludes a compensating effect of the impaired TF expression and therefore underlies the possibility of a potent antithrombotic property of amiodarone in humans, because the balance with TFPI, the physiological antagonist of TF, is critical for thrombus formation.35

Amiodarone regulated TF protein expression at the translational level, because MAP kinase activation, TF mRNA expression, and TF protein degradation remained unaffected. This was confirmed by a metabolic pulse labeling experiment using [15S]-methionine/cysteine as radioactive tracer. Besides its known antiarrhythmic properties, pleiotropic alterations of protein expression by amiodarone have been reported.36–38 A recent report described decreased expression of CACNA1C, the α-1C subunit of the L-Type calcium channel, at the posttranscriptional or translational level.39 Amiodarone also interferes with mitochondrial metabolism and energy supply;40,41 a similar mechanism could explain the impaired TF expression in endothelial cells, because protein translation is energy dependent. Ouabain, an established Na+/K+-ATPase inhibitor, is known to deplete intracellular potassium concentration in endothelial cells and in turn downregulates TF protein expression at the translational level.24,25 Such an effect can be excluded for amiodarone, because it does not affect intracellular potassium levels.

Dealkylation is the first step in degradation of amiodarone and results in the pharmacologically active metabolite MDEA which exhibits plasma levels comparable to amiodarone.23 MDEA is known to exert toxic effects at lower concentrations than amiodarone.42,43 In line with these observations, 10 μmol/L MDEA, but not amiodarone, was toxic for endothelial cells. At lower concentrations, MDEA impaired TF expression and may thus potentiate the antithrombotic effect of amiodarone. MDEA and amiodarone seem to be degraded further, because elevated excretion of free iodine has been observed in humans treated with amiodarone.44 The free iodine is mainly responsible for changes in thyroid function resulting in hypo- or hyperthyroidism;45,46 nevertheless, the iodine is not essential for inhibition of TF protein expression, because amiodarone lacking iodine suppressed TF expression to a similar extent as amiodarone.

About 75% of all SCD are imputable to coronary artery disease.3–5 The majority of them are triggered by ventricular arrhythmia, often caused by acute coronary events.3 This is supported by evidence from autopsies, showing that the majority of SCD are associated to acute coronary thrombosis.6–8 Therefore, in patients with coronary artery disease, it seems likely that acute thrombus formation is the main initial event leading to SCD. In patients suffering from myocardial infarction or congestive heart failure, amiodarone reduced SCD by 29% and total mortality by 13%.20 The present study suggests that amiodarone may decrease SCD not only by preventing ventricular arrhythmia, but also by inhibiting arterial thrombosis. Indeed, subgroup analysis of prophylactic amiodarone treatment did not reveal a greater effectiveness in patients with higher frequencies of ventricular arrhythmia,20 which supports the observation of a antithrombotic pleiotropic effect of amiodarone contributing to the reduction of SCD.

In summary, the present study demonstrates that amiodarone, at clinically relevant concentrations, inhibits arterial
thrombus formation by decreasing TF activity and platelet number in a mouse thrombosis model in vivo. A conserved effect was observed in human vascular cells where amiodarone inhibited TF activity and protein expression at the translational level. Because thrombosis is the main initial event leading to SCD in patients with coronary artery disease, these results reveal new possible insights with respect to the beneficial actions of amiodarone.

Acknowledgments
The authors thank Stephan Keller for expert technical support.

Sources of Funding
This study was supported by the Swiss National Science Foundation (grant no. 3200B0-113328/1 to FCT and grant no. 3100-068118.02/1 to T.F.L.), Bonizzi-Theler Foundation, Velux Foundation, Wolfermann Nägeli Foundation, MERCATOR Foundation, and the Swiss Heart Foundation.

Disclosures
None.

References

Breitenstein et al Amiodarone and Tissue Factor 2237


Amiodarone Inhibits Arterial Thrombus Formation and Tissue Factor Translation
A. Breitenstein, S.F. Stämpfli, G.G. Camici, A. Akhmedov, H.R. Ha, F. Follath, A. Bogdanova, T.F. Lüscher and F.C. Tanner

*Arterioscler Thromb Vasc Biol.* 2008; 28:2231-2238; originally published online October 30, 2008;
doi: 10.1161/ATVBAHA.108.171272

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/28/12/2231

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/10/31/ATVBAHA.108.171272.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at: http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at: http://atvb.ahajournals.org//subscriptions/
Material and Methods

**Carotid artery thrombosis model**

14 week old C57BL/6 mice weighing on average 28g were anaesthetized by intraperitoneal injection of 87 mg/kg sodium pentobarbital (Butler, Columbus, OH). Rose bengal (Fisher Scientific, Fair Lawn, NJ) was diluted to 12 mg/mL in phosphate-buffered saline and then injected into the tail vein at a concentration of 63 mg/kg. Mice were secured in a supine position, placed under a dissecting microscope, and the right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) was applied and connected to a flowmeter (Transonic, Model T106). 6 minutes after rose bengal injection, a 1.5-mW green light laser (540 nm; Melles Griot, Carlsbad, CA) was applied to the site of injury at a distance of 6 cm for 60 minutes or until thrombosis occurred. From the onset of injury, blood flow was monitored up to 120 minutes, at which time the experiment was terminated. Occlusion was defined as flow \( \leq 0.1 \) ml/min for at least 1 minute. Mice were divided into 2 groups: Amiodarone (80 mg/kg with an intraperitoneal injection every 24 hours for 7 days), or vehicle control (1.43% Tween 80, 0.29% benzyl alcohol)

**Amiodarone and MDEA concentration in mouse plasma and thoracic aorta**

Concentrations of amiodarone and MDEA in mouse plasma and thoracic aorta were measured by high performance liquid chromatography [HPLC; LiChrospher® 60, 5C18; 250 x i.d. 4mm; mobile phase: methanol:water:25% NH\(_3\) (89.47:8.77:1.76w/w); flow rate: 1ml/min]. Aorta (25 mg) was homogenized in 0.5 ml methanol at 4°C using a Potter S glass homogenizer (Braun, Melsungen, Germany). After centrifugation at 3000g, the pellet was washed twice with 0.25 ml methanol. The organic extracts were
pooled and evaporated to dryness (Speed Vac, Savant Instruments Inc., Farmingdale, NY). To the residue was dissolved in 75 µl of the mobile, and an aliquot of 50 µl was analyzed. Plasma (200 µl) was mixed with 0.1 ml 0.5 mM KH₂PO₄ and extracted three times with 0.75 ml diethylether. The organic extracts were pooled and evaporated to dryness as described. The residue was dissolved in 100 µl of methanol, and an aliquot of 50 µl was used for HPLC.

PTT, aPTT, tail bleeding time, and blood cell counts

Prothrombin time (PTT) and activated partial thromboplastin time (aPTT) were assessed by the Start4 analyzer (Diagnostica Stago, France) with according reagents (Roche Diagnostics, Switzerland). Plasma from citrated blood (3.2% citrate 1/10) was extracted by 15 minutes centrifugation (2500g, 4°C), and stored immediately at -80°C until analysis. Tail bleeding time was assessed as described²³. Briefly, after anesthetizing mice, the distal 4-mm segment of the tail was removed with a scalpel. Bleeding was monitored by gently absorbing the blood with a filter paper at 15 second intervals without touching the wound. To count blood cells, whole blood was collected in heparin-coated tubes and analyzed by flow cytometry.

Cell Culture

Human aortic endothelial cells (HAECs; Clonetics, Allschwil, Switzerland) and human aortic vascular smooth muscle cells (HAVSMCs; Clonetics) were cultured as described²². Adhering HAECs were grown to confluence and rendered quiescent for 24 hours in medium containing 0.5% FCS before stimulation, whereas HAVSMCs were kept in medium containing 0.2% BSA for 48 hours. Cells were pretreated with amiodarone (Sigma, Buchs, Switzerland), N-monodesethylamiodarone (MDEA), or amiodarone lacking iodine (AMWI). The amiodarone derivatives were prepared by
condensing [2-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)-benzofuran] and [2-butyl-3-(4-hydroxybenzoyl)-benzofuran] with 2-chloroethylamine as previously described\textsuperscript{24}. Their chemical structures were confirmed by \textsuperscript{1}H-NMR, ESI-MS analysis. Cells were stimulated with 5 or 10 ng/ml tumor necrosis factor alpha (TNF-\textalpha; R&D Systems, Minneapolis, MN) or 1 U/ml thrombin (R&D Systems), respectively. Cytotoxicity was assessed by a colorimetric assay to detect lactate dehydrogenase (LDH; Roche, Basel, Switzerland).

Western Blot Analysis

Protein expression was determined by western blot analysis. Antibodies against human TF and tissue factor pathway inhibitor (TFPI; both from American Diagnostica, Stamford, CT) were used at 1:2000 dilution. Antibodies against phosphorylated p38 MAP kinase (p38), p44/42 MAP kinase (ERK), and c-Jun NH2-terminal kinase (JNK; all from Cell Signaling, Danvers, MA) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were diluted to 1:3000, 1:2000, and 1:1000, respectively. The antibody against IkB-\textalpha (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at a 1:1000 dilution. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA) or alpha-tubulin (aT; Sigma) were applied to control for equal protein loading (1:10000 dilution). Primary antibodies were detected with a horseradish peroxidase-linked secondary antibody (Amersham, Munich, Germany).

Real-time PCR

Total RNA was extracted from HAECs with 1 mL TRIzol Reagent (Invitrogen, Lucerne, Switzerland) as described\textsuperscript{12}. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random
hexamer primers (Amersham) in a final volume of 33 µL using 4 µg of RNA. The total cDNA pool obtained served as template for subsequent PCR amplification with primers specific for full-length TF (sense primer: 5'-TCCCCAGAGTTCACACCTTACC–3’, antisense primer: 5'-CCTTTCTCCTGGCCCATACAC–3'; bases 508-529 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)\textsuperscript{25}. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. L28 RNA served as loading control.

**TF activity**

TF surface activity in HAECs and TF activity in mouse carotid artery tissue homogenates was analyzed using a colorimetric assay (American Diagnostica)\textsuperscript{21}. Cells were incubated at 37°C with human FVIIa and FX, allowing for the formation of TF/FVIIa complex at the cell surface. Left carotid arteries from amiodarone treated mice were homogenized in 50 µl lysis buffer (50 mmol Tris-HCl, 100 mmol NaCl, 0.1% Triton X-100 pH 7.4) before incubation. Conversion of FX to FXa was measured by the ability of FXa to cleave a chromogenic substrate. A standard curve was established with lipidated human TF to assure that the results were in the linear range of detection.

**Metabolic labeling**
HAECs were starved, either with or without amiodarone (10 µmol/l) pre-treatment, in FCS-free Dulbecco’s modified Eagle medium (DMEM) lacking methionine and cysteine. After 1 hour, cells were kept in the same medium supplemented with [³⁵S]-methionine/cysteine (100 µCi/ml; Hartmann Analytics, Braunschweig, Germany) and stimulated with TNF-α (5 ng/ml) for 5 hours. TF was immunoprecipitated from cell lysates (150 µl) using a goat anti-human TF antibody (1 µg; R&D Systems) or nonimmune goat IgG (1 µg; Sigma) for 3 hours at 4°C, followed by incubation with protein G sepharose beads (Sigma) for 1 hour under the same conditions. Antibody-protein complexes were washed five times and separated on a 10% SDS-Page. Labelled TF was visualized by Molecular Imager FX (Bio-Rad, Hercules, CA).

**Intracellular potassium concentration**

HAECs were supplemented with ⁸⁶Rb (0.5 µCi/ml) and incubated at 37ºC for 4 hours. Ouabain (1 µmol/L), as a positive control, or amiodarone (10 µmol/L) were added to the cells together with the radioactive tracer. After washing, cells were lysed and the amount of intracellular ⁸⁶Rb was measured using a Packard Tri-Carb 1600 TR liquid scintillation counter (Packard, Meriden, CT). Data were plotted against the incubation time, and the plots fitted as an exponential function \( y = y_0 + a \cdot \exp(-bx) \) using the SigmaPlot analysis module. Uptake of the radioactive tracer was normalized to the amount of protein in cell lysate and related to the radioactivity of the incubation medium.

**Statistical Analysis**
Data are indicated as mean ± SEM. Unpaired Student’s $t$-test was performed for statistical analysis. A $p$ value <0.05 denoted a significant difference.
Table I

Effect of amiodarone on PTT, aPTT, platelet number, and tail bleeding time.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=7)</th>
<th>Amiodarone (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTT [seconds]</td>
<td>11.50±0.11</td>
<td>11.19±0.15</td>
</tr>
<tr>
<td>aPTT [seconds]</td>
<td>20.24±0.79</td>
<td>20.70±0.83</td>
</tr>
<tr>
<td>Total platelet number [x10^3/µl]</td>
<td>1056±22</td>
<td>789±42*</td>
</tr>
<tr>
<td>Tail bleeding time [seconds]</td>
<td>393.0±145.0</td>
<td>349.3±92.3</td>
</tr>
</tbody>
</table>

*P<0.05