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ORIGINAL CONTRIBUTION

Guggulsterone, an anti-inflammatory phytosterol, inhibits tissue factor and arterial thrombosis

Catherine Gebhard
Simon F. Stämpfli
Caroline E. Gebhard
Alexander Akhmedov
Alexander Breitenstein
Giovanni G. Camici
Erik W. Holy
Thomas F. Lüscher
Felix C. Tanner

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C. Gebhard, MD · S.F. Stämpfli, MD
C.E. Gebhard · A. Akhmedov, PhD
A. Breitenstein, MD · G.G. Camici, PhD
E.W. Holy, MD · T.F. Lüscher, MD
F.C. Tanner, MD (✉)
Cardiovascular Research, Institute of Physiology
University of Zurich
Winterthurerstraße 190
8057 Zürich, Switzerland
Tel.: +41-44/635-6469
Fax: +41-44/635-6827
E-Mail: felix.tanner@access.uzh.ch

C. Gebhard, MD · S.F. Stämpfli, MD
C.E. Gebhard · A. Akhmedov, PhD
A. Breitenstein, MD · G.G. Camici, PhD
E.W. Holy, MD · T.F. Lüscher, MD
F.C. Tanner, MD
Center for Integrative Human Physiology
University of Zurich
Zürich, Switzerland

C. Gebhard, MD · S.F. Stämpfli, MD
A. Breitenstein, MD · E.W. Holy, MD
T.F. Lüscher, MD · F.C. Tanner, MD
Cardiology, Cardiovascular Center
University Hospital Zurich
Zürich, Switzerland

Abstract *Background* The phytosterol guggulsterone is a potent anti-inflammatory mediator with less side effects than classic steroids. This study assesses the impact of guggulsterone on tissue factor (TF) expression and thrombus formation. *Methods and results* Guggulsterone inhibited TNF-α-induced endothelial TF protein expression and surface activity in a concentration-dependent manner; in contrast, dexamethasone did not affect TNF-α-induced TF expression. Guggulsterone enhanced endothelial tissue factor pathway inhibitor and impaired plasminogen activator inhibitor-1 as well as vascular cell adhesion molecule-1 protein. Real-time polymerase chain reaction revealed that guggulsterone inhibited TNF-α-induced TF mRNA expression; moreover, it impaired activation of the MAP kinases JNK and p38, while that of ERK remained unaffected. In vivo, guggulsterone inhibited TF activity and photochemical injury induced thrombotic occlusion of mouse carotid artery. Guggulsterone also inhibited TF expression, proliferation, and migration of vascular smooth muscle cells in a concentration-dependent manner. *Conclusions* Guggulsterone inhibits TF expression in vascular cells as well as thrombus formation in vivo; moreover, it impairs vascular smooth muscle cell activation. Hence, this phytosterol offers novel therapeutic options, in particular in inflammatory diseases associated with an increased risk of thrombosis.

Key words arterial thrombosis – photochemical injury – phytosterol – tissue factor

Introduction

Vascular inflammation promotes the progression of coronary artery disease; moreover, it triggers acute coronary syndromes [13, 15]. Tissue factor (TF) is regulated by a variety of inflammatory mediators and initiates activation of coagulation. Consistent with this observation, experimental evidence has revealed an important contribution of TF to arterial thrombosis, the key event in acute coronary syndromes [28, 30, 31]. Clinical observations have indeed confirmed that cytokine concentrations high enough to induce endothelial expression of TF are reached at the culprit lesion of patients with acute coronary syndrome [19].

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Guggulsterone [4,17(20)-pregnadiene-3,16-dione] is a plant derived steroid isolated from the gum resin of the tree *Commiphora mukul*, which has been extensively used in Ayurvedic medicine to treat several conditions associated with inflammation such as hyperlipidemia, obesity, and arthritis [1, 14, 23, 34]. Guggulsterone indeed exerts strong anti-inflammatory effects by suppressing the activation of the transcription factor NF-kappa B in response to different agonists including tumor necrosis factor α (TNF-α) [22]. In line with its anti-inflammatory properties, previous studies revealed that guggulsterone is able to impair LDL oxidation, thereby delaying the progression of atherosclerosis [3, 37].

Since guggulsterone is an anti-inflammatory, well-tolerated, natural drug, this study was designed to investigate its impact on cytokine-induced TF expression as well as arterial thrombus formation in vivo.

Materials and methods

Cell culture

Human aortic endothelial cells (HAECs; Clonetics, Allschwil, Switzerland) and human aortic vascular smooth muscle cells (VSMCs) were cultured as described [24]. THP-1 cells (LGC Promochem, Molsheim, France) were kept in RPMI containing 10% fetal calf serum (FCS). Adhering cells were grown to confluence and rendered quiescent for 24 h before stimulation with 5 ng/ml TNF-α (R&D, Minneapolis, MN). Cells were pretreated with guggulsterone, dexamethasone (all from Sigma, Buchs, Switzerland), or GW-4064 (Tocris Bioscience, Ellisville, MI) for 60 min before stimulation. Cytotoxicity was assessed by a colorimetric assay to detect lactate dehydrogenase release (Roche, Basel, Switzerland) [25].

For proliferation, VSMCs were cultured for 24 h in Dulbecco's modified Eagle medium (DMEM) with 10% FCS. Cells were then serum withdrawn for 48 h before stimulation with 10% FCS in the presence of different guggulsterone concentrations. Media were replaced daily, and cell number was determined after 3 days with a hemocytometer (Assisten, Sondheim, Germany) [5]. For migration, VSMCs were cultured for 24 h in DMEM with 10% FCS and then harvested and resuspended (500,000 cells/ml) for analysis of migration in response to platelet-derived growth factor BB (10 ng/ml) in a modified Boyden chamber system (NeuroProbe Inc, Gaithersburg, MD) [5].

Western blot

Protein expression was determined by Western blot analysis as described [26]. Antibodies against human TF and tissue factor pathway inhibitor (TFPI; both from American Diagnostica, Stamford, CT) were used at 1:2,000 dilution. Antibodies against phosphorylated p38 MAP kinase (p38), p44/42 MAP kinase (extracellular signal-regulated kinase [ERK]), and c-Jun NH2-terminal kinase (JNK; all from Cell Signaling, Danvers, MA) were used at 1:1,000, 1:5,000, and 1:1,000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were used at 1:3,000, 1:2,000, and 1:1,000 dilution, respectively. Antibodies against vascular cell adhesion molecule-1 (VCAM-1; R&D) and plasminogen activator inhibitor-1 (PAI-1; Santa Cruz) were applied at 1:2,000 and 1:4,000 dilution, respectively. Anti-goat glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH; Chemicon, Temecula, CA) was applied to ensure equal protein loading (1:20,000 dilution). Proteins were detected with a horseradish peroxidase-linked secondary antibody (Amersham).

Real-time PCR

All real-time PCR experiments were performed in triplicate using the SYBR Green JumpStart kit (Sigma) in an MX3000P PCR cycler (Stratagene, Amsterdam, The Netherlands). Each reaction (25 µl) contained 2 µl cDNA, 1 pmol of each primer, 0.25 µl of internal reference dye, and 12.5 µl of JumpStart Taq ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody). The following primers were used: human tissue factor (*F3*): sense primer: 5′-TCCCCAGAGTTCACACCTTACC-3′ (bases 508–529 of *F3* cDNA; NCBI no. NM 001993), antisense primer: 5′-TGACCACAAATACCACAGCTCC-3′ (bases 892–913 of *F3* cDNA; NCBI no. NM 001993); human L28: sense primer: 5′-GCATCTGCAATGGATGGT-3′, antisense primer: 5′-TGTTCTTGCGGATCATGTGT-3′. The amplification program consisted of 1 cycle at 95°C for 10 min followed by 40 cycles with a denaturing phase at 95°C for 30 s, an annealing phase at 60°C for 1 min, and an elongation phase at 72°C for 1 min. PCR products were analyzed on an ethidium bromide stained 1% agarose gel, and a melting curve analysis was performed after amplification to verify the accuracy of the amplicon. In each real-time PCR run for F3 and L28, a calibration curve was included that was generated from serial dilutions of the respective purified amplicon [24, 27].

TF activity

Tissue factor activity was analyzed at the surface of HAEC and in mouse carotid artery tissue homogenates, respectively, using a colorimetric assay

(American Diagnostica) according to the manufacturer's recommendations with some modifications as described [5]. Right carotid arteries were homogenized in 50 µl of lysis buffer (50 mmol Tris–HCl, 100 mmol NaCl, 0.1% Triton X-100 pH 7.4) 5 h after oral gavage of guggulsterone (100 mg/kg) or vehicle (0.5% methyl cellulose, 10 ml/kg) and left to stand on ice for 30 min. TF/FVIIa converted factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. A standard curve with lipidated human TF was performed to assure that measurements were taken in the linear range of detection.

Carotid artery thrombosis model

Sixteen weeks old C57BL/6 mice weighing on average 28 ± 1 g were anesthetized by intraperitoneal injection of 2.4 mg sodium pentobarbital (Butler, Columbus, OH) as described [5]. 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 dose of 50 mg/kg. Mice were secured in a supine position, placed under a dissecting microscope (Olympus C-4040 Zoom), 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). After 6 min of 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 min or until thrombosis occurred. From the onset of injury, blood flow was monitored for 120 min, at which time the experiment was terminated. Occlusion was defined as flow ≤0.1 ml/min for at least 1 min [8]. Mice were divided into two groups: guggulsterone (oral gavage, 5 h before surgery; 100 mg/kg guggulsterone diluted in 0.5% methyl cellulose in an injection volume of 10 ml/kg), or vehicle control (0.5 % methyl cellulose).

Statistics

Data are presented as mean ± SEM. Statistical analysis was performed by 2-tailed unpaired Student's t test or ANOVA as appropriate. A probability value of <0.05 was considered significant.

Results

Guggulsterone inhibits TNF-α-induced TF protein expression and activity in vascular cells

Human aortic endothelial cells were stimulated with TNF-α (5 ng/ml) for 5 h in the presence or absence of guggulsterone. Guggulsterone inhibited TNF-α-induced TF expression in a concentration-dependent manner with a maximal effect occurring at 10^{-4} mol/l ($n = 7$; $P < 0.05$; Fig. 1a); similarly, it impaired TNF-α-induced TF surface activity ($n = 4$; $P < 0.05$; Fig. 1b). Guggulsterone also inhibited TF expression in VSMCs ($n = 4$; $P < 0.05$; Fig. 1c), but not in THP-1 cells ($n = 5$; P = NS; Fig. 1d). In contrast to guggulsterone, dexamethasone did not affect TNF-α-induced TF expression ($n = 6$; P = NS; data not shown).

Guggulsterone enhances endothelial TFPI and inhibits PAI-1 as well as VCAM-1 expression

Guggulsterone enhanced basal expression of TFPI in a concentration-dependent manner ($n = 3$; $P < 0.05$; Fig. 2a), but did not affect TNF-α-induced TFPI expression ($n = 3$; P = NS; Fig. 2a). Guggulsterone inhibited basal expression of PAI-1 in a concentration-dependent manner ($n = 7$; $P < 0.05$; Fig. 2b), and a similar effect was observed after TNF-α stimulation ($n = 7$; $P < 0.05$; Fig. 2b). Moreover, guggulsterone inhibited TNF-α-induced VCAM-1 expression in a concentration-dependent and potent manner ($n = 7$; $P < 0.05$; Fig. 2c). In contrast to guggulsterone, dexamethasone only weakly impaired TNF-α-induced VCAM-1 expression (15% inhibition; $n = 3$; $P < 0.05$; data not shown).

Guggulsterone is not toxic for vascular cells

Human aortic endothelial cells and VSMCs were treated with increasing concentrations of guggulsterone or dexamethasone for 6 h. Morphological examination of the cells did not reveal any changes (n = 4–14; data not shown). Moreover, no signs of toxicity were detected by LDH release (n = 4–14; P = NS; data not shown).

Guggulsterone inhibits endothelial TF mRNA expression

Real-time PCR revealed that TF mRNA expression was induced after 2 h of TNF-α stimulation ($n = 4$; $P < 0.05$; Fig. 3). Guggulsterone inhibited TNF-α-induced TF mRNA expression in a concentration-dependent manner ($n = 4$; $P < 0.05$; Fig. 3).

Guggulsterone impairs endothelial TF expression via MAP kinase inhibition

The MAP kinases JNK, p38 and ERK were transiently activated by TNF-α ($n = 5$; Fig. 4a). Guggulsterone

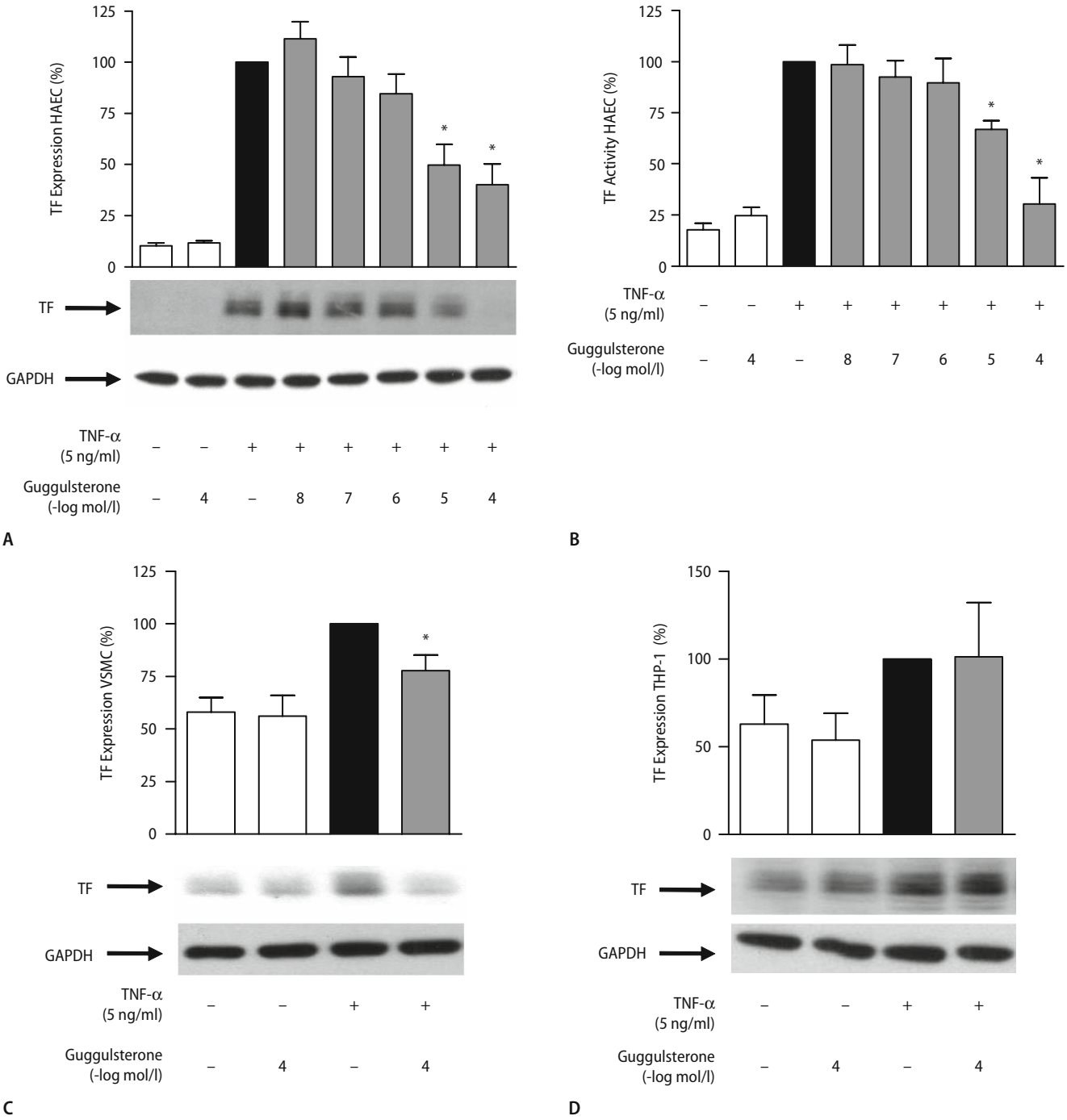

Fig. 1 Guggulsterone inhibits TF protein expression and activity. **a** Guggulsterone inhibits TNF-α-induced TF protein expression of human aortic endothelial cells (HAECs) ($P < 0.05$ vs. TNF-α alone). **b** Guggulsterone inhibits TNF-α-induced TF surface activity of HAECs ($P < 0.05$ vs. TNF-α alone). **c** Guggulsterone inhibits TNF-α-induced TF protein expression of human aortic vascular smooth muscle cells ($P < 0.05$ vs. TNF-α alone). **d** Guggulsterone does not affect TNF-α-induced TF protein expression of THP-I cells (P = NS vs. TNF-α alone). Values are indicated as percent of TNF-α alone; all blots are normalized to GAPDH expression

inhibited phosphorylation of JNK and p38, while that of ERK remained unaffected ($n = 5$; Fig. 4a, b). No significant change in total expression of MAP kinases was observed at any time point with or without guggulsterone. To verify the involvement of MAP kinases in TNF-α-induced TF expression, the

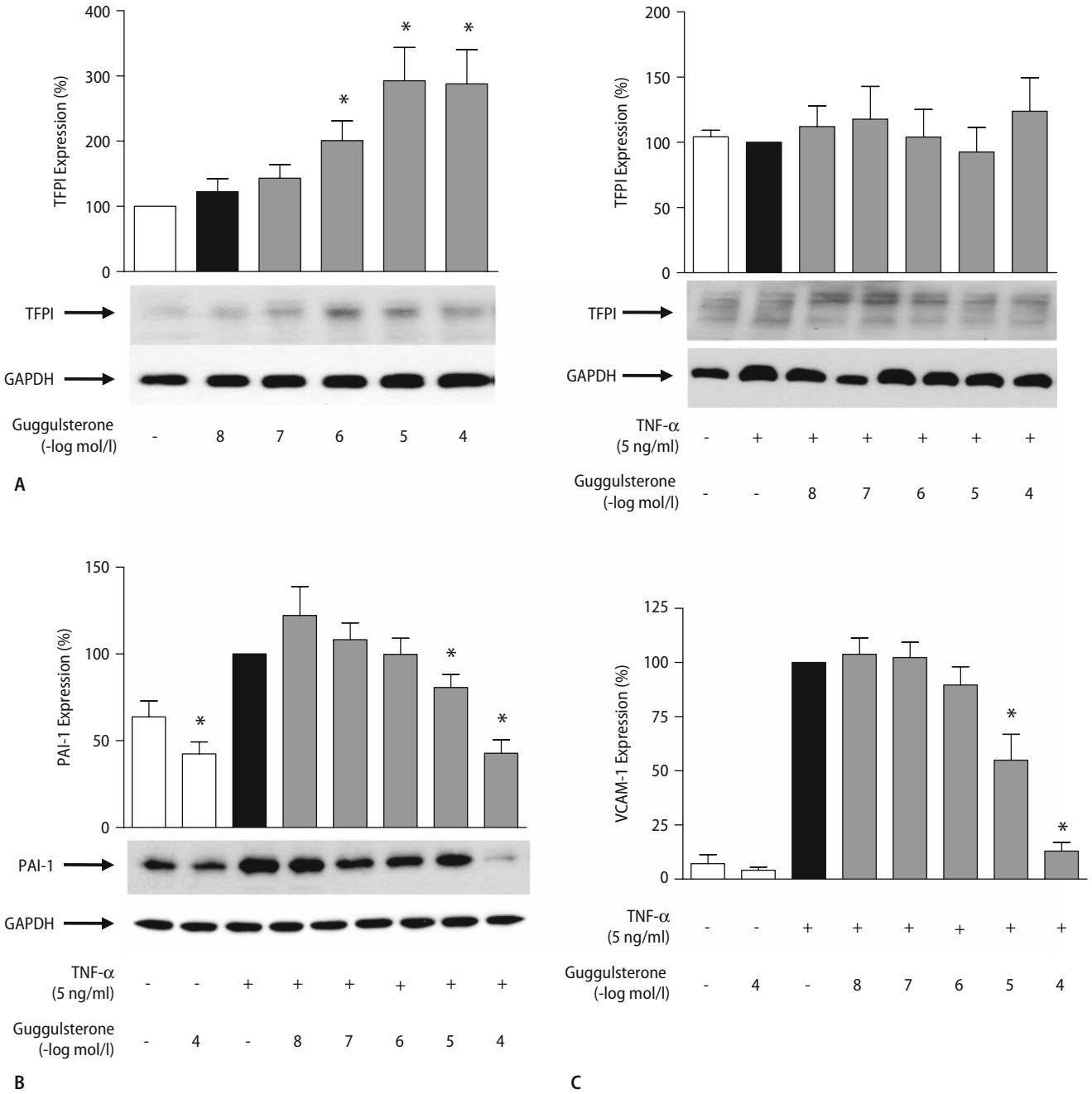

Fig. 2 Guggulsterone enhances endothelial TFPI and impairs PAI-1 as well as VCAM-1 expression. **a** Guggulsterone enhances basal TFPI expression ($P < 0.05$ vs. control), but does not affect TNF-α-induced TFPI expression (P = NS vs. TNF-α alone). **b** Guggulsterone inhibits basal PAI-1 expression ($P < 0.05$ vs. control), and a similar effect is observed after TNF-α stimulation ($P < 0.05$ vs. TNF-α alone). **c** Guggulsterone inhibits TNF-α-induced VCAM-1 expression ($P < 0.05$ vs. control). Values are indicated as percent of control or TNF-α alone; all blots are normalized to GAPDH expression

action of MAP kinase inhibitors was examined. PD98059, SP600125, and SB203580, which specifically inhibit ERK, JNK, and p38, respectively, significantly impaired TF expression after TNF-α stimulation ($n = 6$; $P < 0.05$ for TNF-α alone versus each inhibitor; Fig. 4c).

Farnesoid X receptor (FXR) does not mediate the effect of guggulsterone

Human aortic endothelial cells were stimulated with TNF-α (5 ng/ml) for 5 h after a 1-h treatment with guggulsterone (10^{-4} M) or GW-4064 (10^{-5} M), a

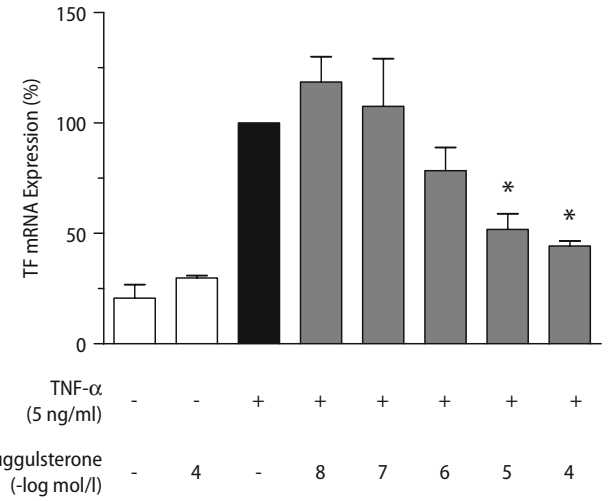

Fig. 3 Guggulsterone inhibits endothelial TF mRNA expression. Real-time PCR reveals that guggulsterone inhibits TNF-α-induced TF mRNA expression ($P < 0.05$ vs. TNF-α alone). Values are indicated as percent of TNF-α alone and normalized to L28 expression

selective and potent FXR agonist. Guggulsterone and GW-4064 both decreased TNF-α-induced endothelial TF protein expression with similar potency ($n = 4$; $^*P < 0.05$). The effect of guggulsterone remained unaltered following treatment with the FXR agonist GW-4064 ($n = 4$; P = NS). No cytotoxic effect of either substance was observed as measured by LDH release (data not shown).

Guggulsterone inhibits TF activity and arterial thrombosis in vivo

Treatment with guggulsterone (100 mg/kg) impaired TF activity in mouse carotid artery in vivo ($n \geq 4$; $P < 0.05$ for guggulsterone vs. vehicle; Fig. 5a). Photochemical injury is an established model of TF depending arterial thrombosis [4]. Initial blood flow in carotid artery of vehicle-treated mice equalled 0.58 ± 0.03 ml/min ($n = 4$); these mice developed thrombotic occlusion within a mean occlusion time of 36.06 ± 2.12 min ($n = 4$; Fig. 5b). Guggulsterone-treated mice exhibited similar initial blood flow (0.67 ± 0.09 ml/min; $n = 7$; P = NS for guggulsterone vs. vehicle), but developed thrombosis within a mean occlusion time of 55.51 ± 4.83 min ($n = 7$; $P < 0.05$ for guggulsterone vs. vehicle; Fig. 5b).

Guggulsterone inhibits proliferation and migration of human VSMCs

Guggulsterone inhibited VSMC proliferation in response to 10% FCS in a concentration-dependent manner by 82% at the maximal concentration ($n = 11$; $P < 0.05$; Fig. 6a). Likewise, guggulsterone inhibited VSMC migration in response to 10 ng/ml platelet-derived growth factor-BB in a concentration-dependent manner by 99% at the maximal concentration ($n = 5$; $P < 0.05$; Fig. 6a).

Discussion

This study demonstrates that the anti-inflammatory phytosterol guggulsterone inhibits TF in HAECs and VSMCs; this effect is related to impaired activation of the MAP kinases JNK and p38 in endothelial cells, resulting in a diminished level of TF mRNA, protein, and activity. Consistent with these observations, guggulsterone inhibits TF activity as well as thrombotic occlusion of mouse carotid artery in vivo. Moreover, guggulsterone impairs proliferation and migration of vascular smooth muscle cells. Hence, guggulsterone may be considered as a novel anti-thrombotic agent for treating inflammatory vascular disorders.

Chronic systemic inflammation, as encountered in rheumatoid arthritis or systemic lupus erythematodes, is associated with a considerably increased risk of cardiovascular disease [13, 35]. TF plays a crucial role in inflammation-induced coagulation as it is upregulated in vascular cells and in vivo by several inflammatory mediators [16, 28, 32]. As a consequence, recent efforts have focused on novel strategies for targeting TF. The phytosterol guggulsterone seems to be a suitable candidate, as it exhibits potent anti-inflammatory and anti-thrombotic properties.

Significant impairment of TF expression in vitro occurred at concentrations as low as 10 μM. Up to now, the pharmacokinetics of guggulsterone in humans remain unknown; however, clinical trials performed in humans indicate that doses up to 6 g per day are safe and well tolerated [29]. This dose correlates well to that applied in our mouse experiments (100 mg/kg bodyweight). A study performed in rats revealed that 50 mg/kg bodyweight oral application of guggulsterone resulted in peak plasma concentrations of 3.2 μM [36]. In view of the above, the guggulsterone concentrations applied in vitro may reasonably well match the plasma concentrations reached in our in vivo experiments.

Activation of MAP kinases mediates TF expression in response to many stimuli [18, 25, 28, 30]. Guggulsterone impaired endothelial TF expression by specifically decreasing phosphorylation of JNK and p38, but not ERK, and consistent with MAP kinase inhibition, guggulsterone promoted a strong reduction in TNF-α-induced TF mRNA. Hence, in endothelial cells, the inhibitory effect of guggulsterone is

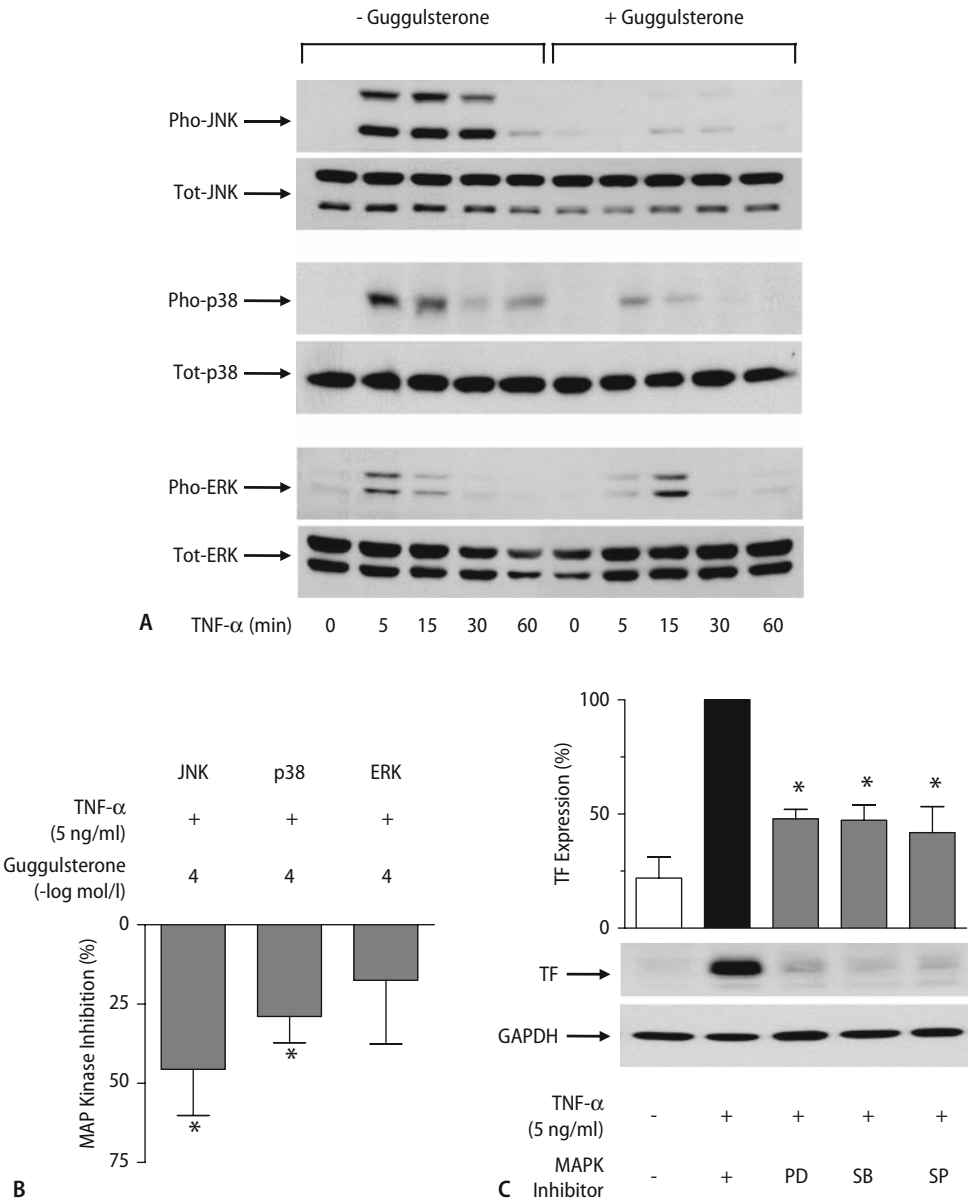

Fig. 4 Guggulsterone impairs endothelial TF expression via MAP kinase inhibition. **a** The MAP kinases JNK, p38, and ERK are transiently activated by TNF-α (5 ng/ml). Guggulsterone inhibits phosphorylation of JNK and p38, but not ERK. No significant change in total expression of MAP kinases is observed. **b** Inhibition of MAP kinase activation by guggulsterone ($P < 0.05$ vs. TNF-α alone). **c** PD98059, SB203580, and SP600125, specifically inhibiting ERK, p38, and JNK, respectively, impair TF expression after TNF-α stimulation ($P < 0.05$ for TNF-α alone vs. each inhibitor)

related to MAP kinase inhibition and occurs at the transcriptional level. Since TNF-α regulates TF expression in vascular smooth muscle cells via activation of MAP kinases as well, its inhibitory effect in this cell type may be similar to that in endothelial cells. This profile of action is remarkably similar to that of dimethylsulfoxide, another inhibitor of TF expression proposed for application on drug-eluting stents [5].

Guggulsterone is an antagonist of the FXR, a key regulator of cholesterol homeostasis [33]. Recent studies demonstrate that FXR is expressed and functional in vascular endothelial cells indicating that FXR may play a role in endothelial homeostasis and serve as a novel molecular target for manipulating vascular inflammation [4, 11, 17]. FXR is activated by bile acids such as chenodeoxycholic acid (CDCA); in addition, synthetic FXR agonists such as GW4064 have been developed. Both guggulsterone and GW-4064 decreased TNF-α-induced endothelial TF expression with similar potency; moreover, the effect of guggulsterone on endothelial TF expression remained unaltered in the presence of GW-4064. These observations suggest that the antithrombotic effects of guggulsterone occur independent of FXR inhibition.

Tissue factor activity is counterbalanced by its endogenous inhibitor TFPI, which is mainly produced by endothelial cells; the balance of these two factors is

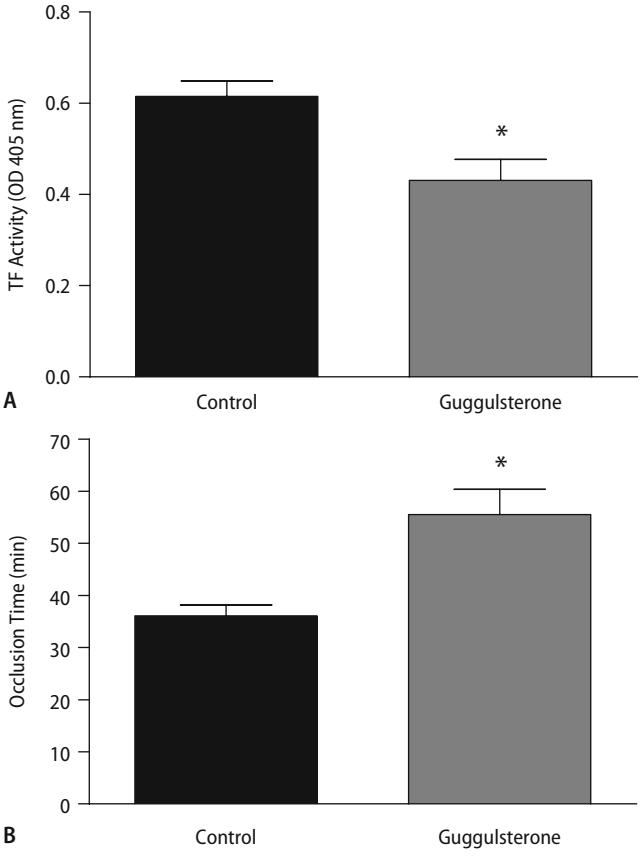

Fig. 5 Guggulsterone inhibits TF activity and thrombotic occlusion in vivo. A Guggulsterone inhibits TF activity in mouse carotid artery ($n = 4–7$; $P < 0.03$). **b** Time to thrombotic occlusion after mouse carotid artery photochemical injury in vivo. Treatment is performed with guggulsterone (100 mg/kg) or vehicle (methyl cellulose), and laser injury is initiated at time = 0 min. Guggulsterone impairs thrombus formation ($n = 4–8$; $P < 0.02$ vs. vehicle)

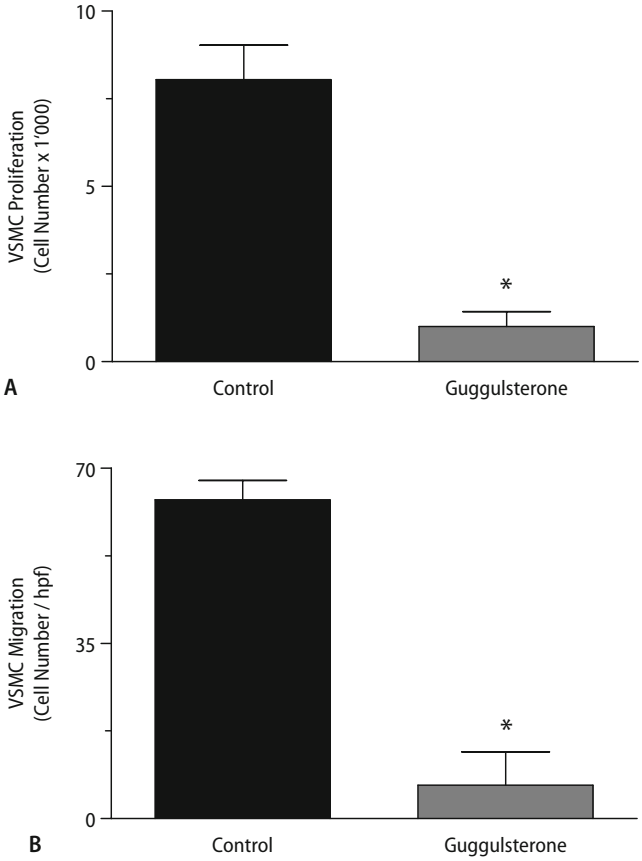

Fig. 6 Guggulsterone inhibits vascular smooth muscle cell activation. **a** Guggulsterone inhibits vascular smooth muscle (VSMC) proliferation ($n = 11$; $P < 0.05$ vs. control). **b** Guggulsterone inhibits vascular smooth muscle (VSMC) migration ($n = 5$; $P < 0.05$ vs. control)

indeed essential in determining thrombus formation [6]. Guggulsterone enhanced basal TFPI expression, which underlines the anti-thrombotic properties of this drug. However, TFPI release remained unaltered in TNF-α stimulated cells, indicating that the effect of guggulsterone on TFPI expression depends on the activation status of the cells. Hence, in an inflammatory environment, the effect of guggulsterone on TFPI expression appears to be overcome by the prothrombotic action of inflammatory mediators. However, under these circumstances, guggulsterone inhibited TF expression, and the lower TF expression in cytokine stimulated cells was not counteracted by a parallel decrease in TFPI; therefore, guggulsterone would be expected to exert potent antithrombotic actions even in an inflammatory environment by reducing TF instead of enhancing TFPI expression. PAI-1 is a serpin suppressing fibrinolysis by inhibiting the activity of tissue plasminogen activator (tPA). PAI-1 levels are elevated in patients with diabetes mellitus and coronary artery disease [2, 21] and are associated with an enhanced risk of coronary events [10, 12]. Besides its effects on TF and TFPI, guggulsterone downregulated PAI-1 expression both in quiescent and TNF-α stimulated cells; hence, guggulsterone may be particularly beneficial in patients with diabetes mellitus.

Photochemical injury was selected for examining thrombus formation because it is an established protocol for TF-dependent arterial thrombosis in vivo [5, 7, 8]. Guggulsterone inhibited thrombotic occlusion, indicating that it may protect from thrombus formation. Indeed, inhibition of TF activity in the mouse carotid artery within 5 h of guggulsterone administration was confirmed and is the most likely explanation for the effect of guggulsterone on thrombus formation.

Migration and proliferation of VSMCs are crucial events involved in neointima formation and restenosis after percutaneous revascularisation. Hence, sub-

stances are applied on drug-eluting stents to reduce restenosis through inhibition of VSMC activation. This study demonstrates that guggulsterone effectively inhibits VSMC proliferation and migration. In addition to VSMC, TF has been implicated in the pathogenesis of neointima formation and stent thrombosis [20, 24, 27]. The pronounced inhibition of both VSMC activation and TF expression renders guggulsterone an attractive candidate for application on drug-eluting stents.

In summary, this study demonstrates that guggulsterone impairs TF in vascular cells and prevents thrombotic occlusion in vivo; further, guggulsterone inhibits VSMC proliferation and migration. These findings may offer new therapeutic approaches to prevent thrombosis in chronic inflammatory disorders or in the context of drug-eluting stents. The known ability of guggulsterone to inhibit platelet aggregation may indeed be very important in this regard [9]. However, further in vivo studies are needed to assess potential therapeutic effects of guggulsterone on neointima formation. Moreover, additional studies need to clarify whether guggulsterone's effects are modulated by differences in genetic background, lifestyle or diet [29]. Only then can specific applications of guggulsterone for the treatment of vascular disease be developed and tested in humans.

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