Tissue factor (TF), a transmembrane glycoprotein, is a major initiator of the extrinsic blood coagulation pathway. Tissue factor binds to factor FVII/FVIIa, and this complex activates factors FIX and FX, leading to generation of thrombin and fibrin deposition. In addition to its prominent role in coagulation, TF-dependent signaling pathways contribute to a variety of pathological processes, including inflammation, atherosclerosis, angiogenesis, and vascular remodeling. Tissue factor is expressed in the vascular wall, whereas monocytes seem to be its major source in circulating human blood. An increased TF expression in monocytes is associated with disseminated intravascular coagulation, acute coronary syndrome, sepsis, and other prothrombotic conditions. Expression of TF is also markedly upregulated in the lung vasculature in pulmonary arterial hypertension, a disease associated with pulmonary vascular remodeling and localized thrombosis. Because TF is an important player in vascular remodeling, inhibition of TF expression could be beneficial to offset the progression of pulmonary arterial hypertension and other cardiovascular diseases.

Earlier studies have indicated an inhibitory effect of cyclic guanosine 3′-5′ monophosphate (cGMP) on expression of TF in monocytes. Cyclic GMP is produced from guanosine-5′-triphosphate through activation of soluble guanylate cyclase (sGC) by nitric oxide leading to vasodilation, as well as inhibition of platelet aggregation and vascular smooth muscle cell proliferation. Nitric oxide–independent agonists of sGC have been recently developed and shown to attenuate systemic and pulmonary hypertension, vascular remodeling, and platelet aggregation in several experimental models.

Objective—Tissue factor (TF), a major initiator of blood coagulation, contributes to inflammation, atherosclerosis, angiogenesis, and vascular remodeling. Pharmacological agonists of soluble guanylate cyclase (sGC) attenuate systemic and pulmonary hypertension, vascular remodeling, and platelet aggregation. However, the influence of these novel pharmacophores on TF is unknown.

Methods and Results—We evaluated effects of BAY 41-2272 and BAY 58-2667 on expression and activity of TF in human monocytes and umbilical vein endothelial cells (HUVECs). Both compounds reduced expression of active TF protein in monocytes stimulated with lipopolysaccharide, as demonstrated by immunoblotting and a TF procoagulant activity assay. In-cell Western assay revealed that this effect was associated with a marked reduction of total and surface TF presentation. Furthermore, BAY 41-2272 and BAY 58-2667 decreased TF protein expression and the TF-dependent procoagulant activity in HUVECs stimulated with TNF-α. The sGC agonists also suppressed transcriptional activity of NF-κB. A siRNA-mediated knockdown of the α1-subunit of sGC in monocytes and HUVECs confirmed that the inhibitory effect of BAY 41-2272 and BAY 58-2667 on TF expression is mediated through the sGC-dependent mechanisms.

Conclusions—Inhibition of TF expression and activity by sGC agonists might provide therapeutic benefits in cardiovascular diseases associated with enhanced procoagulant and inflammatory response. (Arterioscler Thromb Vasc Biol. 2009;29:1578-1586.)

Key Words: tissue factor • procoagulant activity • soluble guanylate cyclase • BAY 41-2272 • BAY 58-2667
expression and procoagulant activity of TF in monocytes stimulated with bacterial lipopolysaccharide (LPS). Similar inhibitory effect of both sGC agonists on TF was also observed in human umbilical vein endothelial cells (HUVECs) stimulated with tumor necrosis factor–α (TNF-α). Furthermore, BAY 41-2272 and BAY 58-2667 suppressed transcriptional activity of NF-κB. Using a small interfering RNA (siRNA)-mediated knockdown of the α1 subunit of sGC in monocytes and endothelial cells, we confirmed that the inhibitory effect of the sGC agonists on TF expression is mediated through the sGC-dependent mechanisms.

**Methods**

For detailed descriptions of the Methods, please see supplemental material available online at http://atvb.ahajournals.org.

**Cell Preparation and Experimental Protocols**

We used a whole-blood system to test the potential of BAY 41-2272 and BAY 58-2667 to attenuate TF activation in monocytes stimulated with LPS. Blood sampling was performed according to the study protocol approved by the Regional Committee for Medical Research Ethics. Informed consent was obtained from 6 healthy volunteers of both sexes (age 24 to 59 years). Human blood was exposed to BAY 41-2272 (1, 10, 100, or 200 μmol/L) or BAY 58-2667 (1, 10, 50, or 100 μmol/L) for 10 or 45 minutes before the LPS challenge. Thereafter, whole blood aliquots were stimulated by adding 5 ng/mL of LPS (strain 026:B6; Difco Laboratories) for 2 hours at 37°C. Mononuclear cells (MNCs) were obtained by density centrifugation using a Lymphoprep solution (Axis-Shield). We also studied the potential of both sGC agonists to inhibit TF activation in human vascular endothelium in a model of resting or TNF-α-stimulated HUVECs (ATCC, Manassas, VA). Forty-eight hours after plating, the cells were starved overnight and exposed to 0.1, 1, 10, 50, or 100 μmol/L of BAY 41-2272 or BAY 58-2667 for 2 hours. Thereafter, HUVECs were stimulated with 10 ng/mL of TNF-α (Sigma-Aldrich) for 4 hours.

**RNA Interference and Nucleofection**

We evaluated whether the effects of BAY 41-2272 and BAY 58-2667 on TF were dependent on the presence of functional sGC by performing experiments in human monocytes or HUVECs with knocked-down expression of the α1 subunit of sGC (sGCα1). For sGCα1-silencing, 3 million monocytes or HUVECs were nucleofected with 3 μg of siRNA using a Nucleofector II device (Lonza Pharmacia Biotech). Viability of MNCs and HUVECs after various treatments was assessed by a dual light reporter gene assay kit (Tropix; Promega) on a Lumoskan RS microplate reader (Labsystems Oy).

**Western Blotting**

TF protein levels in the lysates of isolated monocytes or harvested HUVECs were detected by probing the membranes with a mouse antihuman TF monoclonal antibody (clone TF9 10H10; Calbiochem) and a horseradish peroxidase (HRP)-conjugated goat antimouse secondary antibody (BD Biosciences Pharmingen). Densitometrical readings of TF immunopositive bands were used for statistical comparisons. Protein loading was determined by probing the membranes with a rabbit antihuman β-actin polyclonal antibody (Calbiochem) and a HRP-conjugated goat antirabbit antibody (BD Biosciences Pharmingen). The efficacy of sGCα1 silencing in the RNA interference assay was ascertained by a monoclonal anti-sGCα1 antibody (Abcam), and the expression of TF protein was used as a readout in the sGCα1-knockdown experiments. Levels of IκBα in monocytes and HUVECs were measured by using a mouse antihuman IκBα antibody (Cell Signaling Technology).

**In-Cell Western Assay**

We used the in-cell Western (ICW) assay to characterize total and surface TF levels in resting and stimulated monocytes. Isolated human monocytes were immunostained with mouse antihuman TF (Sigma-Aldrich) antibodies. The signals from primary antibodies were amplified with IRDye800CW-conjugated goat anti-mouse (Rockland Immunochemicals) and Alexa680-conjugated goat antirabbit (Invitrogen) secondary antibodies.

**Flow Cytometry**

After completion of treatments, HUVECs were harvested and immunostained with an Alexa488-conjugated (Alexa-488 Protein Labeling Kit; Invitrogen) mouse antihuman TF monoclonal antibody (Calbiochem). Flow cytometry was performed using a FACSCalibur flow cytometer calibrated with Calibrate beads (BD Biosciences).

**TF Procoagulant Activity**

TF was measured in the intact cells and in frozen/thawed preparations of monocytes using a two-stage clotting assay based on the ability of TF to accelerate the activation of factor X by factor VIIa as previously described.

**Determinations of cGMP and Cell Viability**

Concentrations of cGMP in lysates of MNCs and HUVECs were quantified using a cGMP enzyme-immunoassay kit (Amersham Pharmacia Biotech). Viability of MNCs and HUVECs after various treatments was assessed by flow cytometry analysis of propidium iodide (PI) uptake by cells as previously described.

**Data Analysis**

Each experiment was performed at least 4 times. Samples, where applicable, were assayed in triplicates. Data are expressed as mean±SEM. The treatment effects were tested by ANOVA followed by a Holm-Sidak post hoc test (SigmaStat 3.0; Systat Software). Probability values <0.05 were considered statistically significant.

**Results**

BAY 41-2272 and BAY 58-2667 Inhibit Expression and Activity of TF in Monocytes

We first tested whether the sGC agonists would alter TF protein expression in monocytes, stimulated with LPS, in a whole blood system. A 2-hour LPS exposure resulted in a strong and reproducible elevation of the TF immunopositive band intensity on Western blots (Figure 1, second lanes). Pretreatment with BAY 41-2272 (200 μmol/L) for 10 minutes before stimulation with LPS produced a significant reduction in TF band intensity (Figure 1A). A 10-minute pretreatment with BAY 58-2667 resulted in a more potent reduction of TF band intensity, demonstrating this effect already at the 50-μmol/L concentration.
Increasing the duration of the pretreatment to 45 minutes produced a marked inhibition of TF protein levels starting at 100 μmol/L of BAY 41-2272 and at 10 μmol/L of BAY 58-2667 (Figure 1C and 1D).

Because activation of TF encompasses externalization of TF protein onto the cell surface, we next studied effects of both sGC agonists on levels of surface and total TF in human monocytes using the ICW assay. Stimulation with LPS induced a reproducible increase of surface and total TF protein levels, which were respectively 7- and 10-fold higher than in resting cells (supplemental Figure I). Pretreatment of whole blood with BAY 41-2272 (200 μmol/L for 10 minutes and 100 μmol/L for 45 minutes before LPS) significantly reduced surface and total expression of TF in monocytes, as compared to the LPS-stimulated control cells. Pretreatment with BAY 58-2667 produced a more potent inhibition of surface and total TF expression, because this effect was already observed at the 50-μmol/L concentration (supplemental Figure II).

BAY 41-2272 and BAY 58-2667 attenuated TF protein levels in monocytes via regulation of TF mRNA expression. Specifically, a...
10-minute pretreatment of monocytes with BAY 41-2272 (200 μmol/L) or BAY 58-2667 (50 μmol/L) produced a significant reduction in TF mRNA levels, when compared to the control cells stimulated with LPS alone (supplemental Figure IIIA and IIIB). This inhibitory effect was enhanced after a 45-minute pretreatment with both sGC agonists (supplemental Figure IIIC and IIID).

Because activity of TF is not solely regulated by the presentation of TF antigen on the surface of monocytes, but also via other mechanisms, generalized as the “TF decryption phenomenon,”23 we evaluated its functional activity, using a TF-dependent thrombin generation assay.20 After a 10-minute pretreatment, BAY 41-2272 (200 μmol/L) and BAY 58-2667 (50 μmol/L) inhibited the LPS-induced TF activity in monocytes (Figure 2A and 2B). A 45-minute pretreatment produced a more potent reduction of TF activity in the LPS-stimulated monocytes: BAY 41-2272 exhibited its inhibitory effect at 100 μmol/L, whereas BAY 58-2667 inhibited TF activity already at 10 μmol/L (Figure 2C and 2D).

**BAY 41-2272 and BAY 58-2667 Inhibit Expression and Activity of TF in Human Endothelial Cells**

Because endothelial cells are a source of TF in blood vessels, we used this cell type as a model system to test effects of the sGC agonists on TF. Using flow cytometry of HUVECs immunostained against TF, we found that a 6-hour treatment with BAY 41-2272 (50 and 100 μmol/L) significantly reduced the TF-dependent mean fluorescence intensity (MFI) in resting HUVECs (supplemental Figure IVA and IVB). The population of cells presenting TF on their surface was also reduced (supplemental Figure IVB). In addition, lysates of resting HUVECs contained less TF protein and exhibited a significantly lower TF activity (supplemental Figure IVD and IVE).

Furthermore, we evaluated effects of BAY 41-2272 in HUVECs challenged with TNF-α. TNF-α induced a marked elevation of TF protein expression, an expansion of a TF-positive cell population, and an increase of TF...
BAY 41-2272 (50 and 100 μmol/L) for 2 hours before stimulation with TNF-α resulted in a significant reduction of TF MFI, as compared to the cells stimulated with TNF-α alone (Figure 3A and 3C). The population of TF-positive endothelial cells was also significantly reduced after 100 μmol/L of BAY 41-2272 (50 and 100 μmol/L) reduced TF protein and activity levels in HUVEC lysates (Figure 3D and 3E).

A 6-hour treatment of resting HUVECs with BAY 58-2667 significantly reduced TF MFI and a number of TF-positive cells when applied at concentrations of
10 \mu\text{mol/L} and higher (supplemental Figure VA through VC). We also observed a reduction of TF protein and activity levels in lysates of resting endothelial cells after 50 and 100 \mu\text{mol/L} of BAY 58-2667 (supplemental Figure VD and VE). Pretreatment of HUVECs with BAY 58-2667 (50 and 100 \mu\text{mol/L}) for 2 hours before TNF-\alpha significantly reduced TF MFI and the population of the cells, expressing TF on their surface (Figure 4A through 4C). In addition, BAY 58-2667, at concentrations of 10 \mu\text{mol/L} and higher, attenuated TF protein expression and TF activity levels in lysates of HUVECs stimulated with TNF-\alpha (Figure 4D and 4E).
Inhibition of the TF System by BAY 41-2272 and BAY 58-2667 Is Dependent on the Expression of the α1 Subunit of sGC and Mediated via NF-κB

To evaluate the contribution of functional sGC in the response of the TF system to the sGC agonists, we used a siRNA-mediated knockdown of expression of the α1 subunit of sGC in human monocytes and endothelial cells. As shown in Figure 5, nucleofection of isolated monocytes and HUVECs with the sGCα1-specific siRNA resulted in a profound reduction of endogenous sGCα1 protein expression. Interestingly, TF band intensity was significantly stronger in resting monocytes and HUVECs nucleofected with siRNA against sGCα1 than in the cells nucleofected with control siRNA. Stimulation with LPS and TNF-α elicited an increase in TF protein levels in the cells expressing sGCα1, and this response was enhanced in the cells lacking sGCα1. However, after a knockdown of the sGCα1 expression in monocytes and HUVECs, BAY 41-2272 and BAY 58-2667 lost their potential to attenuate the LPS- or TNF-α-induced TF protein expression, even when applied at the maximal concentrations.

Figure 6 demonstrates that the loss of protein levels of NF-κB in the LPS-stimulated monocytes and the TNF-α-stimulated HUVECs was independent of the sGCα1 expression and was not affected by BAY 41-2272 and BAY 58-2667. A knockdown of sGCα1 resulted in a significantly elevated NF-κB transcriptional activity in monocytes and HUVECs under resting conditions and particularly after stimulation with LPS or TNF-α, respectively. Both sGC agonists markedly attenuated the transcriptional activation of the NF-κB-sensitive reporter in the stimulated cells nucleo-
fected with control siRNA but not in the stimulated cells nucleofected with siRNA against \( \text{sGC/1} \).

**Intracellular cGMP Concentrations**

LPS and TNF-\( \alpha \) significantly increased intracellular cGMP concentrations in monocytes and HUVECs, respectively, nucleofected with control siRNA (supplemental Figure VI). However, no such response occurred in the cells nucleofected with siRNA against \( \text{sGC/1} \). BAY 41-2272 and BAY 58-2667 further increased intracellular cGMP concentrations in stimulated control monocytes and HUVECs but not in the stimulated \( \text{sGC/1} \) knockdown cells.

**Assessment of Cell Viability and Validation of the Anti-TF Antibody**

None of the treatments applied in the present investigation had any significant effect on the fraction of apoptotic cells (supplemental Figure VII). Because most of the data on TF expression was obtained by using a single anti-TF antibody, we tested this antibody specificity by using a siRNA-mediated knockdown of TF expression. The Western blot shown on supplemental Figure VIII illustrates a nearly complete loss of TF immunopositive band in a sample of monocytes nucleofected with the TF-specific siRNA.

**Discussion**

Our investigation reveals that the \( \text{sGC} \) agonists BAY 41-2272 and BAY 58-2667 attenuate protein expression and function of TF in human monocytes stimulated with LPS, as demonstrated by Western blotting, real time RT-PCR, and the TF procoagulant activity assay. This response is associated with a marked reduction of total and surface TF presentation, as assessed by the ICW assay. Furthermore, BAY 41-2272 and BAY 58-2667 reduce TF protein expression in HUVECs, both under resting conditions and after stimulation with TNF-\( \alpha \). This effect is accompanied by a reduction of the TF-dependent procoagulant activity in the lysates of HUVECs under the same experimental conditions. Importantly, the inhibitory effects of both \( \text{sGC} \) agonists on TF expression are dependent on the presence of functional \( \text{sGC} \), because the siRNA-mediated knockdown of the \( \text{\alpha/1} \) subunit of \( \text{sGC} \) render stimulated monocytes and HUVECs insensitive to BAY 41-2271 and BAY 58-2667.

In the present study, BAY 41-2272 and BAY 58-2667 increased intracellular cGMP concentrations and decreased the expression of active TF protein, as well as the surface TF presentation in human monocytes and vascular endothelial cells stimulated, respectively, with LPS and TNF-\( \alpha \). These data are in agreement with an earlier investigation demonstrating an inhibitory role of cyclic nucleotides in regulation of TF expression in monocytes.\(^{10}\) Importantly, proinflammatory mediators such as LPS per se have been shown to increase intracellular levels of cGMP,\(^ {24}\) as we also found in the present study. Apparently, further elevation of cGMP levels through pharmacological stimulation of \( \text{sGC} \) produces a self-limiting negative regulation of the inflammatory response. Of note, the observed changes in TF expression and functional activity in monocytes and HUVECs could not be attributed to the differences in the survival of cells after various treatments, as the fraction of apoptotic cells remained unchanged.

The concentrations of the \( \text{sGC} \) agonists used to achieve an acute pharmacological response in our in vitro investigation were higher than the concentrations previously tested to produce hemodynamic responses in vivo.\(^ {12–14}\) However, an important finding was that the effects of BAY 41-2271 and BAY 58-2667 in monocytes were time-dependent, as both compounds elicited a stronger inhibitory activity on TF when the exposure time before the LPS stimulation was extended from 10 to 45 minutes. Thus, it is likely that with chronic administration the effective concentrations of these compounds required to elicit a significant inhibitory effect on TF in vivo would be much lower.

In humans, the promoter of \( F3 \) gene contains a binding site for NF-\( \kappa B \), which mediates responses to TNF-\( \alpha \) and LPS.\(^ {25,26}\) Although TNF-\( \alpha \) and LPS can regulate TF expression by virtue of posttranscriptional stabilization of TF mRNA,\(^ {27,28}\) upregulation of TF expression in response to inflammatory stimuli is largely dependent on the transcriptional activity of NF-\( \kappa B \). In the present study, we demonstrate that inhibition of TF by the \( \text{sGC} \) agonists in both monocytes and HUVECs is mediated at the level of the transcriptional regulation of NF-\( \kappa B \) and not upstream of \( \text{IkBa} \), because the loss of \( \text{IkBa} \) protein after stimulation with LPS and TNF-\( \alpha \) was not affected by BAY 41-2272 and BAY 58-2667. Our findings are in agreement with Pan et al, who have reported that YC-1, a nonspecific nitric oxide–independent stimulator of \( \text{sGC} \), reduced the LPS-induced expression of inflammatory cytokines in mononuclear cells via inhibition of NF-\( \kappa B \).\(^ {29}\) Similarly, attenuation of the LPS-induced NF-\( \kappa B \) activity by YC-1 has been recently shown in microglial cells.\(^ {30}\)

In circulating blood monocytes, regulation of TF expression and functional activity depends on interactions with other cell types, notably platelets. Platelets play a crucial role in facilitation of the LPS-induced TF expression in monocytes.\(^ {31}\) Furthermore, platelets promote thrombin generation by providing a catalytic surface for the TF/FVIIa/FXa complex and release FVa,\(^ {32}\) and can also affect monocyte TF expression by releasing platelet-derived factors and inducing TNF-\( \alpha \).\(^ {20,33,34}\) The cross-talk between monocytes and platelets results in synthesis and surface exposure of various adhesion molecules, such as P-selectin, lymphocyte function-associated antigen-1, membrane-activated complex-1,\(^ {35}\) and CD40 with its CD40-ligand, which enhance platelet-monocyte interactions and TF expression by monocytes.\(^ {36,37}\) Interestingly, BAY 41-2272 has been recently shown to inhibit platelet activity\(^ {16}\) and down-regulate the expression of P-selectin,\(^ {38}\) supporting our findings that these novel pharmacophores may exert antithrombotic effects by reducing TF expression in circulating blood monocytes and vascular endothelial cells.

The presence of functional \( \text{sGC} \) is crucial for BAY 41-2272 and BAY 58-2667 to elicit the inhibitory effect on TF expression, because the siRNA-mediated knockdown of the \( \alpha/1 \) subunit of \( \text{sGC} \) completely negated this effect. Interestingly, TF protein levels were significantly higher in resting monocytes and HUVECs in which \( \text{\text{sGC/1}} \) expression was knocked-down as compared to the resting cells nucleofected with control siRNA. Likewise, the TF expression was also greatly enhanced in the \( \text{sGC/1} \)-deficient cells after stimulation with LPS and TNF-\( \alpha \). These findings indicate that in human monocytes and endothel-
lial cells sGC plays an important regulatory role by controlling the TF system. Furthermore, the downregulation of TF expression and functional activity by BAY 41-2272 and BAY 58-2667 are mediated through the sGC-dependent mechanisms involving the suppression of transcriptional activity of NF-κB. Taken together, our data strongly suggest that inhibition of TF expression and activity by sGC agonists might provide therapeutic benefits in cardiovascular diseases associated with enhanced prothrombotic and inflammatory responses.

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Disclosures

J.P.S. is a full-time employee of Bayer Schering Pharma AG and a coinventor in several patent applications on sGC agonists.

References

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SUPPLEMENTAL MATERIALS

SOLUBLE GUANYLATE CYCLASE AGONISTS INHIBIT EXPRESSION AND PROCOAGULANT ACTIVITY OF TISSUE FACTOR
Mikhail A. Sovershaev, Elena M. Egorina, John-Bjarne Hansen, Bjarne Østerud, Pál Pacher, Johannes-Peter Stasch, and Oleg V. Evgenov

METHODS

Cell preparation and experimental protocols
Blood sampling was performed according to the study protocol approved by the Regional Committee for Medical Research Ethics. Informed consent was obtained from six healthy volunteers of both sexes (age 24-59 yrs). Venous blood was collected using plastic syringes and 19-G needles into sterile polystyrene tubes (BD Biosciences Pharmingen, Franklin Lakes, NJ) containing heparin (Sigma-Aldrich, Munich, Germany) at a final concentration of 10 U/ml.

We utilized a whole blood system to test the potential of the soluble guanylate cyclase (sGC) agonists, BAY 41-2272 and BAY 58-2667, to attenuate tissue factor (TF) activation in monocytes stimulated with bacterial lipopolysaccharide (LPS). Sampled blood was exposed to BAY 41-2272 (1, 10, 100, or 200 µM) or BAY 58-2667 (1, 10, 50, or 100 µM) for 10 or 45 min prior to the LPS challenge. Thereafter, 1-ml aliquots of whole blood were stimulated by adding 5 ng/ml of LPS (strain 026:B6; Difco Laboratories, Detroit, MI) for 2 hrs in a rotary incubator (180 rpm) at 37°C. The LPS stimulation was terminated by adding disodium ethylenediamine tetraacetic acid (EDTA; Merck, Darmstadt, Germany) at a final concentration of 5 mM. Blood was then diluted by adding one volume of sterile normal saline, carefully layered over a Lymphoprep solution (Axis-Shield, Oslo, Norway), and subjected to density centrifugation at 415 g for 15 min. Mononuclear cells (MNC) were washed once, counted on a Sysmex K1000 (TOA
Medical Electronics, Kobe, Japan), pelleted, and kept frozen at –20°C until analyzed for TF procoagulant activity and protein levels.

We also studied the efficacy of both sGC agonists to inhibit TF activation in human vascular endothelium in a model of resting or tumor necrosis factor-α (TNF-α)-stimulated human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA). HUVEC were grown in a RPMI full growth medium supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, Munich, Germany) in a humidified 5% CO₂/air incubator. Forty-eight hrs after plating, the cells were starved from FCS overnight and exposed to 0.1, 1, 10, 50, or 100 µM of BAY 41-2272 or BAY 58-2667. Two hrs after exposure, HUVEC were stimulated with TNF-α (Sigma-Aldrich, Munich, Germany) at a concentration of 10 ng/ml for 4 hrs. At the end of the stimulation, the cells were harvested for further analysis of TF procoagulant activity and protein levels.

**sGCα1 RNA interference**

We investigated whether the effects of BAY 41-2272 and BAY 58-2667 on TF were dependent on the presence of functional sGC by performing experiments in human monocytes or HUVEC with knocked-down expression of the α1 subunit of sGC (sGCα1). Three million Lymphoprep-isolated monocytes were nucleofected with 3 µg of small interfering RNA (siRNA) with the sense sequence 5’-AUAUGUUACGAGGAAGAUG-3’ using a Nucleofector II device (Lonza Cologne, Cologne, Germany) according to the manufacturer's instructions. Control monocytes were nucleofected with scrambled control siRNA. Following electrical pulse, 500 µl of the CO₂- and temperature-equilibrated nucleofection medium was immediately added to neutralize the nucleofection solution. The cells were seeded into 12-well plates and kept in a water-jacketed cell culture incubator for further use. For assessment of nucleofection efficiency in the sGCα1-silencing experiments, 1 µg of the green fluorescent protein (GFP)-encoding plasmid pGFP-C1 was used. Thereafter, monocytes were reintroduced to whole blood samples, which were previously depleted for MNC \(^1\), and the highest effective concentrations of BAY 41-2272 or BAY
58-2667 preceding the LPS stimulation were applied as described above. Similarly, three million HUVEC were nucleofected with siRNA, plated and treated as described above after a recovery period. The quality of the anti-TF antibody used in this study was controlled in a TF knock-down experiment, in which HUVEC were nucleofected with TF-specific siRNA with the sense sequence 5′-GCGCUUCAGGCACUACAAA-3′. Forty eight hrs after nucleofection, the cells were stimulated with TNF-α, harvested, and kept frozen for further western blotting analysis.

All reagents and materials were screened for contamination by LPS with a CoaTest according to the manufacturer’s instructions (Haemochrom Diagnostica, Frederiksberg, Denmark).

**Western blotting**

Pellets of isolated monocytes or harvested HUVEC were lysed in an ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), and a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were briefly sonicated and centrifuged at 10,000 g for 15 min at 4°C. Supernatants were mixed with a SDS sample buffer, electrophoresed on 10% polyacrylamide gels and electroblotted onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). Membranes were blocked in 5% skimmed milk and probed with a mouse anti-human TF monoclonal antibody at a 1:1,000 dilution (clone TF9 10H10; Calbiochem, San Diego, CA) and a HRP-conjugated goat anti-mouse antibody at a 1:2,000 dilution (BD Biosciences Pharmingen, Franklin Lakes, NJ). Detection and quantification of immunopositive bands were performed using a Lumilmager F1 and LumiAnalyst software (Boehringer Mannheim, Mannheim, Germany). Stripping of membranes was done by incubation in 0.2 M NaOH for 10 min before washing and re-blocking with 5% skimmed milk. Protein loading was determined by probing the membranes with a rabbit anti-human β-actin polyclonal antibody at a 1:2,000 dilution (Calbiochem, San Diego, CA) and a HRP-conjugated goat anti-rabbit antibody at a 1:2,000 dilution (BD Biosciences Pharmingen,
Franklin Lakes, NJ). The efficacy of sGCα1 silencing was ascertained by immunoblotting lysates of monocytes or HUVEC with a monoclonal anti-sGCα1 antibody (Abcam, Cambridge, MA), and the expression of TF protein was used as a readout in the sGCα1-knock-down experiments. Levels of IκBα in monocytes and HUVEC were measured by using a mouse anti-human IκBα antibody (Cell Signaling Technology, Danvers, MA).

**In-Cell Western assay**

We utilized the In-Cell Western assay to characterize total and surface TF levels in monocytes. This assay allows comparison of levels of the antigen of interest in their cellular context in plated fixed cells \(^2\). Following treatments, monocytes were prepared as described above, plated for 30 min on a 96-well plate in RPMI medium, fixed with 4% paraformaldehyde, permeabilized with methanol to evaluate total (intracellular and surface) TF or left intact to evaluate only surface TF, blocked with 3% goat serum, and immunostained with anti-TF (Calbiochem, San Diego, CA) and anti-glyceraldehydephosphate dehydrogenase (GAPDH; Sigma-Aldrich, Munich, Germany) antibodies. The signal from the mouse anti-human TF monoclonal antibody was detected with IRDye800CW-conjugated goat anti-mouse polyclonal antibodies (1.25 µg/ml; Rockland Immunochemicals, Rockland, PA). The signal from the rabbit anti-GAPDH polyclonal antibody was detected with Alexa680-conjugated goat anti-rabbit polyclonal antibodies (1 µg/ml; Invitrogen, Carlsbad, CA). After washing, images of Alexa680 and IRDye800CW fluorescence were obtained on 700-nm and 800-nm channels of an Odyssey infrared imager (LI-COR Biosciences, Bad Homburg, Germany). For statistical analysis, integrated intensities of fluorescence in wells were processed using software provided with the imaging station.
**TF procoagulant activity**

TF was measured in the intact cells and in frozen/thawed preparations of monocytes using a two-stage clotting assay based on the ability of TF to accelerate the activation of factor X by factor VIIa as previously described \(^3\).

**Flow cytometry**

Following completion of treatments, HUVEC were harvested, fixed with 4% paraformaldehyde, blocked with 3% goat serum and immunostained with an Alexa-conjugated (Alexa-488 Protein Labeling Kit; Invitrogen, Carlsbad, CA) mouse anti-human TF monoclonal antibody (Calbiochem, San Diego, CA) at a concentration of 10 µg/ml for 45 min on ice. Simultest \(\gamma 2a/\gamma 1\) (BD Biosciences Pharmingen, Franklin Lakes, NJ) was used as an isotype control. After immunostaining, cells were washed once, re-suspended in PBS with 0.1% BSA (Sigma-Aldrich, Munich, Germany) and kept on ice until further analysis.

Flow cytometry was performed using a FACSCalibur flow cytometer calibrated with Calibrate™ beads (BD Biosciences, Franklin Lakes, NJ). Background fluorescence for isotype control was set at less than 2% of the stained cells. The fluorescence intensities of at least 5,000 events were recorded. Data acquisition and analysis were done using a CellQuest software (BD Biosciences, Franklin Lakes, NJ).

**Real time RT-PCR**

Total RNA was isolated from cell pellets using an RNeasy Plus Mini Kit (Qiagen Norge, Oslo, Norway) followed by complementary DNA (cDNA) synthesis with High-capacity cDNA Reverse Transcription Kit. Real-time PCR was performed on a 7900HT Fast Real-Time PCR System using TaqMan Fast Universal PCR MasterMix. Tissue factor mRNA expression was analyzed by using forward primer 5’-CCCCAGAGTTCACACCTTACCT-3’, a reverse primer 5’-CACTTTTGTTCCCCACCTGTCA-3’, and a probe 6-FAM-5’-
AGACAAACCTCGGACAGCCAACAATTCA-3'-BHQ-1. For assessment of an irrelevant housekeeping gene, we analysed an expression of cyclophilin using forward 5'-GTACTATTAGCCATGGTCAACCCC-3' and a reverse 5'-CAGTCAAAGGAGACGCGGCC-3' primer, and a probe 6-FAM-5'-AGACAAACCTCGGACAGCCAACAATTCA-3'-BHQ-1. The results were analyzed on a Sequence Detection System (v2.2.1). All reagents for reverse-transcriptase reaction and real-time PCR were purchased from Applied Biosystems (Foster City, CA).

**Nuclear factor-kappa B (NF-κB) reporter gene assay**

Five million MNC were isolated from fresh whole blood by the Lymphoprep density centrifugation and nucleofected with 3 µg of plasmid DNA. The pNF-κB-conA-LUC reporter plasmids (Clontech Laboratories, Mountain View, CA) feature a firefly luciferase gene driven by a promoter sensitive to NF-κB 4. Nucleofection efficiency was monitored by using pCMV-bgal plasmid constitutively expressing β-galactosidase. The Amaxa Nucleofector II device was used together with a Human Monocyte Nucleofector kit according to the manufacturer’s instructions for plasmid DNA delivery (Lonza Gologne, Cologne, Germany). Similarly, pNF-κB-conA-LUC and pCMV-bgal plasmids were nucleofected into HUVEC using the Amaxa Nucleofector II together with a HUVEC nucleofector kit (Lonza Gologne, Cologne, Germany).

After nucleofection, the cells were seeded into 24-well plates and kept in a 5% CO2/air incubator at 37°C. Following a 6-hr stabilization period, the cells were treated with the highest effective concentrations of BAY 41-2772 or BAY 58-2667 for 45 min prior to stimulation with LPS or for 2 hrs prior to stimulation with TNF-α. Thereafter, the cells were harvested into the lysis buffer. Luciferase and β-galactosidase activities were assessed by a dual light reporter gene assay kit (Tropix; Promega, Madison, WI) on a Lumoskan RS microplate reader (Labsystems Oy, Vantaa, Finland).
Measurements of intracellular cGMP concentrations

Concentrations of cGMP in lysates of MNC and HUVEC were quantified using a cGMP enzyme-immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Briefly, following various treatments, one million cells were harvested and centrifuged at 100 g for 10 min at 4°C. The pellets were re-suspended in 250 μl of a lysis reagent and shaken for 10 min at room temperature. Following centrifugation at 1,000 g for 3 min at 4°C to remove the debris, cell lysates were used for the cGMP assay. Intracellular cGMP content was expressed in femtomoles per one million cells.

Assessment of cell viability

Viability of MNC and HUVEC after various treatments was assayed by flow cytometry analysis of propidium iodide (PI) uptake by cells as previously described. Briefly, upon completion of treatments one million MNC or HUVEC were washed once in ice-cold PBS and transferred to 1 ml of PBS containing freshly dissolved PI at final concentration of 20 μg/ml. Cell were incubated for 20 min at 37°C, then washed once in PBS, and the PI-positive population was analyzed using the FACSCalibur cytometer (BD Biosciences, Franklin Lakes, NJ). Cells were gated from the debris using a forward scatter channel against a side scatter channel acquisition, and fluorescence of PI was recorded on the FL2 channel. A sample of cells, not stained with PI, was used to establish a PI-negative cell population. Data acquisition and analysis were done using the CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Data analysis

Each experiment was performed at least four times. Samples, where applicable, were assayed in triplicates. Data are expressed as mean ± SEM. The treatment effects were tested by ANOVA
followed by a Holm-Sidak post hoc test (SigmaStat 3.0; Systat Software, Richmond, CA). Probability values <0.05 were considered statistically significant.

REFERENCES


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. BAY 41-2272 inhibits the LPS-induced surface and total TF expression in human monocytes. Plots illustrate dose-dependent reductions in surface (A and C) and total (B and D) TF protein expression in the LPS-stimulated monocytes after pretreatment with BAY 41-2272 for 10 or 45 min. AU, arbitrary units. * P < 0.05 vs. the LPS-stimulated cells.

Supplemental Figure II. BAY 58-2667 inhibits the LPS-induced surface and total TF expression in human monocytes. Plots illustrate dose-dependent reductions in surface (A and C) and total (B and D) TF protein expression in the LPS-stimulated monocytes after pretreatment with BAY 58-2667 for 10 or 45 min. AU, arbitrary units. * P < 0.05 vs. the LPS-stimulated cells.

Supplemental Figure III. BAY 41-2272 and BAY 58-2667 inhibit the LPS-induced TF mRNA expression in human monocytes. Plots illustrate dose-dependent reductions in the LPS-induced TF mRNA levels when whole blood aliquots were pre-treated with BAY 41-2272 (A and C) or BAY 58-2667 (B and D) for 10 or 45 min prior to LPS. * P < 0.05 vs. the LPS-stimulated cells.

Supplemental Figure IV. BAY 41-2272 inhibits TF expression and functional activity in resting human endothelial cells. TF-Alexa488 mean fluorescence intensities (MFI) (A), populations of TF-presenting cells (B, C), TF protein expression (D), and TF procoagulant activity (E) in resting HUVEC treated with BAY 41-2272 for 6 hrs. Plot on the panel D represents changes in the TF band densities relative to non-treated cells. WB, western blotting. Data are mean ± SEM. * P < 0.05 vs. non-treated cells.
**Supplemental Figure V. BAY 58-2667 inhibits TF expression and functional activity in resting human endothelial cells.** TF-Alexa488 mean fluorescence intensities (MFI) (A), populations of TF-presenting cells (B, C), TF protein expression (D), and TF procoagulant activity (E) in resting HUVEC treated with BAY 58-2667 for 6 hrs. Plot on the panel D represents changes in the TF band densities relative to non-treated cells. WB, western blotting. Data are mean ± SEM. * P < 0.05 vs. the untreated cells.

**Supplemental Figure VI. Intracellular cGMP concentrations in monocytes and HUVEC.** Stimulation of monocytes (A) and HUVEC (B) nucleofected with control siRNA with LPS and TNF-α, respectively, increased intracellular cGMP levels. Pretreatment of control monocytes and HUVEC with BAY 41-2772 or BAY 58-2667 prior to stimulation with LPS or TNF-α further increased intracellular cGMP concentrations. However, no increase in intracellular cGMP levels occurred in cells nucleofected with siRNA against sGCα1. * P < 0.05 vs. resting cells; † P < 0.05 vs. the LPS- or TNF-α-stimulated cells.

**Supplemental Figure VII. Fractions of apoptotic cells.** Rates of apoptosis in monocytes (A-D) and HUVEC (E, F) following different treatments including BAY 41-2272, BAY 58-2667, LPS or TNF-α.

**Supplemental Figure VIII. Specificity of the anti-TF antibody.** Specificity of the anti-TF antibody was confirmed by the loss of TF immunopositive band in a sample of HUVEC transfected with anti-TF siRNA. Plot represents changes in the TF band density relative to cells nucleofected with control siRNA. WB, western blotting.
Supplemental Figure I.
Supplemental Figure II.
Supplemental Figure III.
Supplemental Figure IV.
Supplemental Figure V.
Supplemental Figure VI.
Supplemental Figure VII.
Supplemental Figure VIII.