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Abstract

Berberine (BBR) is a novel natural hypolipidemic agent. This study investigates whether BBR, similar to statins, exerts pleiotropic effects on endothelial tissue factor (TF) expression. BBR enhanced tumor necrosis factor-alpha (TNF-alpha) and thrombin induced TF expression in human endothelial cells by 3.5-fold. These effects were paralleled by an enhanced TF surface activity. In contrast, expression of TF pathway inhibitor was impaired. BBR enhanced TNF-alpha induced TF mRNA expression; however, TF promoter activity was inhibited. Activation of ERK and p38 remained unaffected, while c-Jun terminal NH(2) kinase was inhibited. BBR reduced TF mRNA degradation rates, prolonging its half-life from 1.1 to 4.3 h. The HMG-CoA reductase inhibitor simvastatin impaired thrombin induced TF expression, and BBR blunted this inhibition. Simvastatin did not affect TNF-alpha induced TF expression, and BBR enhanced TF under these conditions. Administration of BBR (100 mg/kg/d) increased TF activity and impaired TFPI expression in carotid artery of ApoE(-/-) mice. BBR enhances TF via mRNA stabilization at clinically relevant concentrations. Clinical application of BBR, either as an alternative to or in combination with statins, should be considered with caution.
Berberine, a Natural Lipid-Lowering Drug, Exerts Prothrombotic Effects on Vascular Cells

Role of Tissue Factor mRNA Stabilization

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Word count: 4799

Short title: Berberine enhances tissue factor

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Abstract

**Aims.** Berberine (BBR) is a novel natural hypolipidemic agent. This study investigates whether BBR, similar to statins, exerts pleiotropic effects on endothelial tissue factor (TF) expression.

**Methods and Results.** BBR enhanced tumor necrosis factor-α (TNF-α) and thrombin induced TF expression in human endothelial cells by 3.5-fold. These effects were paralleled by an enhanced TF surface activity. In contrast, expression of TF pathway inhibitor was impaired. BBR enhanced TNF-α induced TF mRNA expression; however, TF promoter activity was inhibited. Activation ERK and p38 remained unaffected, while c-Jun terminal NH₂ kinase was inhibited. BBR reduced TF mRNA degradation rates, prolonging its half-life from 1.1 to 4.3 hours. The HMG-CoA reductase inhibitor simvastatin impaired thrombin induced TF expression, and BBR blunted this inhibition. Simvastatin did not affect TNF-α induced TF expression, and BBR enhanced TF under these conditions. Administration of BBR (100 mg/kg/d) increased TF activity and impaired TFPI expression in carotid artery of ApoE⁻/⁻ mice.

**Conclusions.** BBR enhances TF via mRNA stabilization at clinically relevant concentrations. Clinical application of BBR, either as an alternative to or in combination with statins, should be considered with caution.

**Key Words:** Berberine, Tissue Factor, Tissue Factor Pathway Inhibitor, mRNA Stability, Simvastatin
Introduction

Arterial thrombosis is the critical event in acute coronary syndromes, peripheral ischemia, and stroke. Tissue factor (TF) plays an essential role in coagulation by binding factor VII, which activates factor X, finally leading to thrombin generation [1]. TF expression is detected in a variety of cell types within the atherosclerotic vessel wall and is induced by inflammatory mediators such as TNF-α, histamine, or lipopolysaccharide [2,3,4,5]. Elevated levels of TF are indeed present in plaques from patients with unstable angina and enhance plaque thrombogenicity; further, an involvement of TF in drug-eluting stent thrombosis has been discussed as well [6,7,8]. Tissue factor pathway inhibitor (TFPI) acts as the direct endogenous inhibitor of the TF/FVIIa complex [9]. Increasing evidence indicates that modulation of the physiological balance between TF and TFPI has an important impact on thrombus formation [10,11].

Berberine (BBR), an alkaloid isolated from the Chinese herb huanglian (Coptis chinensis), has been extensively used in traditional Chinese medicine. Recently, BBR was identified as a promising lipid-lowering drug, potently upregulating hepatic low-density lipoprotein (LDL) receptor expression. This effect occurred via LDL receptor mRNA stabilization rather than increased promoter activity and required extracellular signal regulated kinase (ERK) activation [12]. Further studies revealed that the BBR induced LDL receptor mRNA stabilization involved novel regulatory proteins located downstream of the ERK pathway and able to interact with sequences in the proximal section of
the LDL receptor mRNA 3’ untranslated region (UTR) [13].

Since these observations suggest a therapeutic application of BBR, either as a monotherapy or in combination with statins, this study addressed the question whether BBR, similar to statins, exerts pleiotropic effects on endothelial TF expression.
Methods

Cell Culture

Human aortic endothelial cells (HAEC; Clonetics, Allschwil, Switzerland) were cultured as described [3,4]. Adhering cells were grown to confluence in 3 cm dishes and rendered quiescent in medium supplemented with 0.5% fetal bovine serum for 24 hours before stimulation with 5 ng/mL TNF-α (R&D Systems, Minneapolis, MN) or 1 U/ml thrombin (Sigma, St. Louis, MO). Cells were treated with BBR (Cayman Chemical, Ann Arbor, MI), simvastatin (Sigma), or both for 60 min prior to stimulation. Cytotoxicity was assessed with a colorimetric assay for detection of lactate dehydrogenase release (Roche, Basel, Switzerland).

Western Blot

Protein expression was determined by Western blot analysis as described [3,4]. Antibodies against human TF and TFPI (both from American Diagnostica, Stamford, CT) were used at 1:2000 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 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, ERK, and JNK (all from Cell Signaling) were used at 1:2000, 1:5000, and 1:1000 dilution, respectively. All blots were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (1:5000 dilution, Chemicon International, Temecula, CA).
**TF Surface Activity**

TF activity at the surface of HAEC was analyzed using a colorimetric assay (American Diagnostica) [3,4]. TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. TF activity was measured against a standard curve performed with lipidated human TF to assure that measurements were taken in the linear range of detection.

**Real Time PCR**

Total RNA was extracted from HAEC using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Biosciences, Piscataway, NJ) in a final volume of 33 µl using 4 µg of cDNA. Real time PCR was performed in an MX3000P PCR cycler (Stratagene, Amsterdam, The Netherlands) as described [7,8]. All experiments were performed using the SYBR Green JumpStart kit (Sigma). 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: for full length human TF (F3): sense 5’-TCCCCAGAGTTTCACACCTTACC-3’, antisense 5’-CCTTTTCCTGCGGCCATACAC-3’ (bases 843-863 of F3 cDNA; NCBI no. NM 001993); for human ribosomal L28: sense 5’-GCATCTGCAATGGATGGT-3’, antisense 5’-
CCTTTCTCCTGGCCCATACAC-3’. The amplification program consisted of 1 cycle at 95˚C for 10 min, followed by 35 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. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for TF and L28, a calibration curve was included that was generated from serial dilutions of purified amplicons.

**TF Promoter Activity**

The TF promoter (-227 bp to +121 bp) was inserted upstream of the luciferase cDNA and the SV40 PolyA signal into the multiple cloning site of the helper vector VQAd5K-NpA (provided by ViraQuest Inc., North Liberty, IA). In a first step, HindIII and BamHI restriction sites of VQAd5K-NpA were used to insert a 2.7 kb HindIII/BamHI restriction fragment of pGL2-Basic vector (Promega, Madison, WI) containing the luciferase cDNA and the SV40 PolyA signal. In a second step, a 0.3 kb Kpnl restriction fragment from a human TF promoter plasmid including the TF minimal promoter [14] kindly provided by Dr. Nigel Mackman (University of North Carolina, Chapel Hill; NC) was ligated into the Kpnl site of the resulting construct. The whole insert was sequenced to confirm its orientation and the absence of any nucleotide substitutions. This construct named VQAd5/hTF/Luc was used for production of an adenoviral vector (Ad5/hTF/Luc). For transduction, the vector was added to HAEC at an moi of 60 pfu/cell for 1 hour and then removed. HAEC were kept in growth
medium for 24 hours and then serum-starved for 24 hours prior to TNF-α stimulation with or without BBR (30 µmol/L) for 5 hours. Firefly luciferase activity was determined in cell lysates using a luminometer (Berthold Technologies, Bad Wildbad, Germany). Protein concentration of the cell lysates was determined for normalization of luciferase activity.

**TF mRNA Stability**

2 hours after TNF-α stimulation, transcription was stopped by addition of actinomycin D (Sigma, St. Louis, MO) at a concentration of 10 µg/ml. At the indicated time points following addition of actinomycin D, cells were washed with PBS and immediately lysed with TRIzol reagent for RNA isolation. RNA was processed for real-time PCR analysis as described above. Values were then plotted on a logarithmic scale as a function of time, and the half-life was calculated using linear equation [15].

**In vitro RNA decay assay**

A 297-bp PCR fragment from 3’-UTR of the human TF cDNA was subcloned into the BamHI and XhoI restriction sites of pBluescript II KS (+) (Stratagene). This construct was linearized with KpnI and used as a template for generation of a 390-nt riboprobe labeled with 50 µCi [α-32P]UTP (25 Ci/mmol, Perkin Elmer) using T7 RNA polymerase (Promega) and m7G5’pppG (cap). The labeling reaction was performed for 1 h at 30°C followed by removal of the DNA template through incubation with RQ1 RNase-Free DNase for 15 min at 37°C. The riboprobe was then purified on 6% Tris boric acid-EDTA buffer-urea
polyacrylamide gel. Cytoplasmic proteins were extracted in lysis buffer (25 mM HEPES, pH 7.6, 5 mM MgCl₂, 1.5 mM KCl, 2 mM DTT, 1 mM PMSF, 0.1% NP-40). ³²P-labeled riboprobe (10⁵ cpm) was added to 20 µg cytoplasmic protein extracts (1 µg protein/µl) in buffer (20 mM HEPES (pH 7.0), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1mM PMSF), and this mixture incubated at room temperature in RNA-binding buffer (20 µl) containing 20 mM HEPES (pH 7.0), 20% glycerol, 100 mM KCl, 3 mM MgCl₂, 2 mM DTT, 0.5 % NP-40, yeast RNA (1 µg), and heparin (1 µg). Aliquots were removed at 0 min, 15 min, 30 min, and 60 min time-points and placed in 100 µl stop solution containing 25 mM Tris (pH 7.6), 400 mM NaCl, 0.1 % SDS, and yeast RNA (10 µg). Samples were extracted with phenol-cloroform, precipitated with ethanol, and separated by gel-electrophoresis on 5% polyacrylamide gel containing 7M urea. Bands were visualized by autoradiography using a phosphoimager.

**In vivo study**

For analysis of TF and TFPI in vivo, 10 week old male ApoE⁻/⁻ mice (C57BL6, Jackson Laboratories, Bar Harbor, ME) weighing an average of 28g were fed a normal chow diet (KLIBA NAFAG, Kaiseraugst, Switzerland) and treated with BBR at a dose of 100 mg/kg/d orally for 10 days. *Control ApoE⁻/⁻ mice matched for age, sex, and weight received an equal volume of vehicle (0.9% saline).* Mice were then euthanized and the left common carotid artery harvested for analysis of TF activity and TFPI expression. Analysis of TF activity in the arterial homogenates was performed using a colorimetric assay (American Diagnostica). TFPI expression was assessed by ELISA (American Diagnostica).
Statistics

Data are presented as mean±SEM. Statistical analysis was performed by ANOVA or 2-tailed unpaired Student t-test as appropriate. A value of p<0.05 was considered significant. All results are representative of at least 4 independent experiments.
Results

Berberine enhances TF protein expression and surface activity

HAEC were stimulated with TNF-α (5 ng/ml) or thrombin (1 U/ml) for 5 hours. 1 hour pretreatment with BBR (1-30 µmol/l) enhanced TNF-α induced TF expression in a concentration-dependent manner with an EC50 value of 2 µmol/l (n=5; p<0.01; Figure 1A); the maximal effect occurred at 30 µmol/l and resulted in a 3.4-fold higher expression as compared to TNF-α alone. Similarly, BBR enhanced thrombin induced TF expression by 3.3-fold as compared to thrombin alone (n=5; p<0.05; Figure 1B). BBR did not alter TF expression under basal conditions (n=10; p=NS; Figure 1A and B). The effect of BBR on TF expression was paralleled by an enhanced TF surface activity, which was 1.6 times higher than that by TNF-α alone (n=4; p<0.0005; Figure 1C). BBR did not alter TF surface activity under basal conditions (n=4; p=NS; Figure 1C).

BBR (1-30 µmol/l) inhibited TFPI expression of TNF-α stimulated HAEC by 45±15% as compared to TNF-α alone with an EC50 value of 5.7 µmol/l (n=4; p<0.05; Figure 1D). Similarly, BBR impaired basal TFPI expression by 52±12% (n=4; p<0.005; Figure 1D).

LDH release was not affected by any BBR concentration used (n=4; p=NS; data not shown) indicating that BBR did not exert cytotoxic effects.

Berberine enhances TF mRNA level

Real-time PCR revealed that TNF-α induced TF mRNA expression within 2 hours of stimulation (n=5; p<0.0001; Figure 2A). BBR (30 µmol/l) enhanced
TNF-α induced TF mRNA expression by 1.6-fold as compared to TNF-α alone (n=5; p<0.05; Figure 2A).

**Berberine inhibits TF promoter activity**

To assess whether the effect on TF mRNA was mediated by enhanced transcription, the impact of BBR on TF promoter activity was analyzed after transfection of HAEC with a plasmid expressing firefly luciferase under control of the human TF promoter (-221bp to +121bp). TNF-α enhanced TF promoter activity by 2.9-fold as compared to control conditions (n=4; p<0.0001; Figure 2B). Treatment with BBR (30 µmol/l) impaired promoter activation (n=4; p<0.001; Figure 2B). BBR did not affect basal promoter activity (n=4; p=NS; Figure 2B).

**Berberine inhibits JNK phosphorylation**

To determine whether inhibition of TF promoter activity was paralleled by inhibition of MAP kinases, phosphorylation of these mediators was assessed at different time points after TNF-α stimulation. TNF-α (5 ng/mL) transiently activated JNK, ERK, and p38; maximal activation was observed after 15 minutes and returned to basal levels within 60 minutes (Figure 3A). Treatment with BBR inhibited phosphorylation of JNK by 48±12% as compared to TNF-α alone (n=4; p<0.05; Figure 3B), while phosphorylation of ERK and p38 was not significantly reduced (n=4; p=NS; Figure 3B). Total expression of JNK, ERK, and p38 remained unaffected at all time points examined (Figure 3A).
**Berberine stabilizes TF mRNA**

TF mRNA was quantified by real-time PCR at different time points after addition of the inhibitor of transcription, actinomycin D. BBR (30 µmol/L) delayed TF mRNA degradation, increasing the half-life of TF mRNA from 64 min under control conditions to 260 min in the presence of BBR (n=5; Figure 4A). To study the role of the 3'-untranslated region (3'-UTR), which includes five AU-rich elements known to mediate TF mRNA stability, an in vitro RNA decay assay was performed. A $^{32}$P-radiolabeled 390 nt transcript containing the last 297 nt of the TF 3'-UTR was incubated with cytoplasmatic extracts generated from endothelial cells either untreated or treated with TNF-α, berberine, or TNF-α+berberine. Analysis of transcript decay by densitometry showed no significant difference in transcript degradation rate between the different groups (n=4; p=NS; Figure 4B).

**Berberine abrogates the inhibitory action of statins on TF expression**

Statins exert pleiotropic anti-thrombotic effects through inhibition of TF expression in different cell types including endothelial cells [16,17,18]. Since statins and BBR exhibit additive lipid-lowering effects, the impact of such a combination on endothelial TF protein expression was examined. Simvastatin (1 µmol/l) inhibited thrombin induced TF expression by 40±14% as compared to thrombin alone (n=5; p<0.05; Figure 5A), while such an effect was not observed in TNF-α stimulated endothelial cells (n=4; p=NS for TNF-α + simvastatin vs TNF-α alone; Figure 5B). BBR antagonized the inhibitory effect of simvastatin on thrombin induced TF expression (n=5; p<0.05 for simvastatin + BBR vs
simvastatin alone; p=NS for simvastatin + BBR vs BBR alone; Figure 5A). In both thrombin and TNF-α stimulated HAEC, treatment with simvastatin had no effect on the enhanced TF expression occurring in the presence of BBR (n=4; p=NS; Figure 5B).

**BBR enhances TF and inhibits TFPI in vivo**

The effect of berberine on expression of TF and TFPI was assessed in ApoE−/− mice. Animals were treated for 10 days with berberine (100 mg/kg/d). The left common carotid artery was harvested for analysis of TF activity and TFPI expression. TF activity was enhanced by nearly 2-fold in the berberine treated group (n=7; p<0.05; Figure 6A). This increase in TF activity was paralleled by a 43.5% decrease in TFPI expression as compared to control (n=7; p<0.05; Figure 6B).
Discussion

This study demonstrates that BBR, at clinically relevant concentrations stimulating hepatic LDL receptor expression [12], enhances TNF-α and thrombin induced endothelial TF expression via stabilization of TF mRNA. Moreover, BBR inhibits endothelial TFPI expression, and abrogates the inhibitory effects of statins on endothelial TF. Similar effects on TF and TFPI are observed in ApoE-/- mice treated with BBR. These observations suggest that application of BBR as a lipid-lowering medication, either alone or in combination with a statin, may favor the development of thrombosis, in particular in the inflammatory environment of atherosclerotic lesions [19].

As endothelial cells are situated at the luminal surface of vessels and thereby exposed to circulating FVII, modulation of TF expression at the endothelial surface can have major consequences for thrombus formation. BBR enhanced TNF-α and thrombin induced TF expression, suggesting that it promotes thrombus formation under both inflammatory and prothrombotic conditions. Since TF is inhibited by TFPI, the balance of these factors is essential for vascular homeostasis [9,10,20]. In addition to enhancing TF, BBR impaired basal as well as TNF-α induced endothelial TFPI expression, which represents an additional prothrombotic potential. These effects of BBR on TF and TFPI may be particularly relevant in acute coronary syndromes, which are usually triggered by vascular inflammation leading to thrombus formation.

The BBR concentrations applied in vitro (1 to 30 µmol/l) are comparable to those used in another study [12], where concentrations ranged from 0.5 to 15
µg/ml corresponding to 1.3 to 40 µmol/l. The pharmacokinetics of berberine in humans are barely understood and still need to be characterized. However, a study performed in patients with congestive heart failure reported that oral administration of 1.2 g BBR per day led to plasma concentrations of 190±0.08 ng/ml (0.5±0.03 µmol/l) [21]. For the in vivo animal studies performed by others as well as for our own in vivo experiments, BBR was administrated orally at a dose of 50 and 100 mg/kg/d corresponding to an oral intake of 3 and 6 g BBR per day, respectively, for an adult weighing 60 kg [12]; hence, BBR intake was higher than in the study performed in humans, suggesting that BBR plasma concentrations may well reach values of 2 µmol/l under these conditions. The berberine mediated effects on endothelial TF were statistically significant at concentrations as low as 1 µmol/l, and consistent with a biologically relevant concentration range, the in vivo effects of BBR on TF and TFPI were similar to those observed in human cells.

The MAP kinases JNK, ERK, and p38 are critically involved in mediating transcriptional regulation of TF expression in response to both TNF-α and thrombin [1]. Although BBR enhanced TF mRNA levels, it inhibited activation of JNK without affecting that of ERK and p38, and, in line with this observation, impaired TF promoter activation. These data indicate that BBR upregulates TF mRNA by a post-transcriptional mechanism able to counteract the impaired TF promoter activation. Analysis of TF mRNA decay rates indeed confirmed that BBR prolonged the half life of TF mRNA from 64 to 260 minutes, highlighting the crucial role of mRNA stability in the regulation of TF expression. A similar effect on the half life of TF mRNA was observed with dexamethasone in human
monocytes [5,22]. The importance of mRNA stability in mediating TF expression, however, varies depending on the stimulus involved; indeed, LPS induces TF mainly by enhancing mRNA stability rather than increasing the rate of transcription, while the opposite effect accounts for the action of PMA [23]. Hence, it is conceivable that the mechanism of action of BBR on endothelial TF expression differs depending on the stimulus activating the endothelium.

In mammalian cells, AU sequences regrouped to AUUUA pentamers in so-called AU-rich elements (ARE) located in the 3’ untranslated region of mRNA are known to modulate mRNA decay [12,24,25]. ARE have been described in the 3’-UTR of human TF mRNA and are involved in TF mRNA turnover [22,26]. Analysis of the decay rates of a 390 nt transcript containing the last 297 nt of the TF 3’-UTR suggested that the stabilizing effect of berberine on TF mRNA is not mediated by elements within the last 297 nt of the TF 3’-UTR since there was no difference in degradation rates of the transcript in the presence of cytoplasmatic extracts from cells treated with or without BBR. 3’-UTR ARE and their binding proteins are not the only regulators of mRNA stability; other mechanisms such as inhibition of translational elongation or the presence of stabilizing sequences located in the 5’-UTR can modulate mRNA decay as well [23,24]. So far, however, it remains unknown whether the latter mechanisms are involved in regulating TF mRNA stability.

Statins are among the most-widely prescribed drugs, and the indications for their prescription have been extended over the years. Besides their well established lipid-lowering properties, statins exert cardio-protective pleiotropic effects [27,28]. Simvastatin indeed inhibited thrombin induced endothelial TF
expression; however, TNF-α induced TF expression was not affected, indicating that such pleiotropic effects are modulated by the mediators involved in endothelial activation. Since another statin, cerivastatin, prevented TNF-α induced TF expression in human endothelial cells [29], this class of drugs may be heterogenous with regard to modulation of TF expression; alternatively, it should be considered that this experiment was performed in human umbilical vein endothelial cells and that experimental conditions might vary. BBR blunted the inhibitory effect of simvastatin on thrombin induced TF expression; moreover, it enhanced TF expression in cells treated with either TNF-α alone or TNF-α together with simvastatin. Both effects can be explained by the posttranscriptional mechanisms through which BBR enhances TF expression.

In summary, this study reveals that the natural lipid-lowering drug, BBR, enhances TF expression and in parallel decreases TFPI expression both in human endothelial cells and in arteries of ApoE^{-/-} mice. Hence, BBR may favor the development of thrombus formation, particularly in patients with atherosclerotic lesions and those with acute coronary syndromes, since prothrombotic and inflammatory environments are often encountered under these conditions. Therefore, clinical application of BBR should be considered with caution, at least until large-scale clinical trials have proven its safety.
Acknowledgements

This study was supported by Swiss National Science Foundation (grant no. 3200B0-113328/1 to FCT and grant no. 3100-068118.02/1 to TFL), Velux Foundation, Wolfermann Näge1 Foundation, and the Swiss Heart Foundation.
References


Figure 1

A

B

C

D

TF expression (%)

TF → GAPDH

TNF-α - + + + + +

BBR (µM) - - 30 1 3 10 30

TF activity (%)

TNF-α - - + + +

BBR (30µM) - + - - +

TFPI expression (%)

TFPI → GAPDH

TNF-α - + + + + +

BBR (µM) - - 30 1 3 10 30

*  **  ***
Figure 2

A

![Graph showing TF/L28 mRNA expression (%) for different treatments of TNF-α and BBR (30µM).]

B

![Graph showing TF promoter activity (%) for different treatments of TNF-α and BBR (30µM).]
**Figure 3**

**A**

- BBR  |  + BBR

Pho-JNK  |  
Total-JNK  |  
Pho-ERK  |  
Total-ERK  |  
Pho-p38  |  
Total-p38  |  

TNF-α (minutes) 0 5 15 30 60 0 5 15 30 60

**B**

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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>BBR (30μM)</td>
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<td>+</td>
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MAP kinase inhibition (%)
Figure 4

A

TF mRNA expression (%)

TF Transcript (% of T0)

0 30 60 90 120

Minutes

TNF-α (t1/2 = 64 min)

TNF-α + BBR (t1/2 = 260 min)

B

TF Transcript (% of T0)

Control

TNF-α

BBR

TNF-α + BBR

0 30 60 90 120

Minutes
Figure 5

A

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B

<table>
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<th>TF expression (%)</th>
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Figure 6

A

TF activity (OD 405 nm)

Control | BBR

B

TFPI antigen (ng/ml)

Control | BBR
Figure Legends

Figure 1: BBR enhances TF protein expression and surface activity, and inhibits TFPI expression

Western blot analysis demonstrates that BBR enhances TNF-α (A) and thrombin (B) induced TF protein expression. *p<0.01 vs TNF-α alone, *p<0.05 vs thrombin alone. C. BBR enhances TNF-α induced TF surface activity. *p<0.001 vs TNF-α alone. D. BBR inhibits TFPI protein expression. *p<0.005 vs control; **p<0.05 vs. TNF-α alone. All blots are normalized to GAPDH expression.

Figure 2: BBR enhances TF mRNA expression, but inhibits TF promoter activation.

A. Real-time PCR demonstrates that BBR enhances TNF-α induced TF mRNA expression. Values are normalized to L28 expression. *p<0.005 vs TNF-α alone. B. BBR inhibits TNF-α induced TF promoter activation. Values are normalized to total protein concentration. *p<0.001 vs TNF-α alone.

Figure 3: BBR inhibits JNK phosphorylation.

A. TNF-α leads to transient phosphorylation (Pho) of JNK, ERK, and p38. BBR inhibits activation of JNK, but not ERK and p38. Total (tot) expression of JNK, ERK, and p38 remains unchanged. B. BBR inhibits phosphorylation of JNK after 15 minutes of TNF-α stimulation. *p<0.05 vs TNF-α alone.
Figure 4: BBR stabilizes TF mRNA

A. The decay of TF mRNA after addition of actinomycin D is monitored by real time PCR and normalized to L28 mRNA. Normalized TF mRNA levels are plotted in a semi-logarithmic scale as percentage of mRNA remaining versus time. The half-lives of TF mRNA are indicated. B. In vitro RNA decay assay reveals that the 3’UTR is not involved in the effect of BBR on TF mRNA. p=NS vs control.

Figure 5: BBR abrogates TF inhibition by simvastatin

A. Simvastatin inhibits thrombin induced TF expression as determined by western blot. **p<0.05 vs thrombin alone. BBR abrogates this effect. *p<0.05 vs thrombin alone, *p<0.05 vs thrombin + simvastatin. B. Simvastatin does not affect TNF-α induced TF expression. p=NS vs TNF-α alone. BBR enhances TF expression in the presence of simvastatin. *p<0.05 vs TNF-α alone, *p<0.05 vs TNF-α + simvastatin.

Figure 6: BBR enhances TF and inhibits TFPI in vivo

A. BBR enhances TF activity in ApoE-/ mouse. *p<0.05 vs control. B. BBR inhibits TFPI expression in ApoE-/ mouse. *p<0.05 vs control.