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Original article

Berberine, a natural lipid-lowering drug, exerts prothrombotic effects on vascular cells

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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-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 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-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. 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.

1. 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.

2. Methods

2.1. 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 h 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).

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2.2. 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).

2.3. 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.

2.4. 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′–TCCCCA-GAGTTCACACCTTACC–3′, antisense 5′–CCTTTCTCCTGGCCCATACAC–3′ (bases 843–863 of F3 cDNA; NCBI no. NM 001993); for human ribosomal L28: sense 5′–GCATCTGCAATGGATGGT–3′, antisense 5′–CCTTTCTCCTGGCCCAT-ACAC–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.

2.5. 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 KpnI restriction fragment from a human TF promoter plasmid including the TF minimal promoter [14] kindly provided by Dr. Nigel Mackman (University of North Carolina, Chapell Hill; NC) was ligated into the KpnI 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 h and then removed. HAEC were kept in growth medium for 24 h and then serum-starved for 24 h prior to TNF-α stimulation with or without BBR (30 μmol/L) for 5 h. 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.

2.6. TF mRNA stability

2 h 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].

2.7. 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-^{32}P]UTP (25 Ci/mmol, Perkin Elmer) using T7 RNA polymerase (Promega) and m^{7}G5′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). ^{32}P-labeled riboprobe (10^5 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, 1 mM 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 $MgCl_2$, 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–chloroform, precipitated with ethanol, and separated by gel-electrophoresis on 5% polyacrylamide gel containing 7 M urea. Bands were visualized by autoradiography using a phosphoimager.

2.8. 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 28 g 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).

2.9. 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.