Reciprocal Regulation of Endothelial Nitric-Oxide Synthase and NADPH Oxidase by Betulinic Acid in Human Endothelial Cells

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ABSTRACT

Nitric oxide (NO) produced by endothelial NO synthase (eNOS) is a protective principle in the vasculature. Many cardiovascular diseases are associated with reduced NO bioactivity and eNOS uncoupling due to oxidative stress. Compounds that reverse eNOS uncoupling and increase eNOS expression are of therapeutic interest. Zizyphi Spinosi semen (ZSS) is one of the most widely used traditional Chinese herbs with protective effects on the cardiovascular system. In human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells, an extract of ZSS increased eNOS promoter activity, eNOS mRNA and protein expression, and NO production in a concentration- and time-dependent manner. Major ZSS constituents include sapoines, such as jujuboside A and B, and pentacyclic triterpenes, such as betulin and betulinic acid. Jujuboside A, jujuboside B, or betulin had no significant effect on eNOS expression, whereas betulinic acid increased eNOS mRNA and protein expression in HUVEC and EA.hy 926 cells. Interestingly, betulinic acid also attenuated the expression of NADPH oxidase subunits Nox4 and p22phox, thereby reducing oxidative stress and improving eNOS function. Consequently, betulinic acid-treated endothelial cells showed an increased production of bioactive NO (as indicated by a higher efficacy in stimulating cGMP generation in RFL-6 reporter cells). Thus, betulinic acid possesses combined properties of eNOS up-regulation and NADPH oxidase down-regulation. Compounds such as betulinic acid may have a therapeutic potential in cardiovascular disease.

Nitrates and NO donors, such as sodium nitroprusside and nitroglycerin, are potent vasodilators and lower blood pressure by activating the NO-cGMP pathway. These compounds have also been shown to reduce vascular smooth muscle cell proliferation and to ameliorate atheromas in experimental models. A number of compounds with pronounced vasodilator and anti-atherosclerotic properties have been isolated from natural sources. Many cardiovascular diseases are associated with reduced NO bioactivity and eNOS uncoupling due to oxidative stress. Compounds that reverse eNOS uncoupling and increase eNOS expression are of therapeutic interest. Zizyphi Spinosi semen (ZSS) is one of the most widely used traditional Chinese herbs with protective effects on the cardiovascular system. In human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells, an extract of ZSS increased eNOS promoter activity, eNOS mRNA and protein expression, and NO production in a concentration- and time-dependent manner. Major ZSS constituents include saponins, such as jujuboside A and B, and pentacyclic triterpenes, such as betulin and betulinic acid. Jujuboside A, jujuboside B, or betulin had no significant effect on eNOS expression, whereas betulinic acid increased eNOS mRNA and protein expression in HUVEC and EA.hy 926 cells. Interestingly, betulinic acid also attenuated the expression of NADPH oxidase subunits Nox4 and p22phox, thereby reducing oxidative stress and improving eNOS function. Consequently, betulinic acid-treated endothelial cells showed an increased production of bioactive NO (as indicated by a higher efficacy in stimulating cGMP generation in RFL-6 reporter cells). Thus, betulinic acid possesses combined properties of eNOS up-regulation and NADPH oxidase down-regulation. Compounds such as betulinic acid may have a therapeutic potential in cardiovascular disease.
found to be up-regulated; however, the up-regulated enzyme turned out to be dysfunctional (Hink et al., 2001; Mollnau et al., 2002). A dysfunctional eNOS produces reactive oxygen species (ROS) at the expense of NO (Xia et al., 1998). This has been referred to as eNOS “uncoupling” (Förstermann and Munzel, 2006). The main reason for eNOS uncoupling is a deficiency of the essential eNOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄) (Channon, 2004). Oxidation of BH₄ due to NADPH oxide-mediated vascular oxidative stress may represent a major cause of BH₄ deficiency in many cases (Förstermann and Munzel, 2006). Suppression of oxidative stress by down-regulating the expression or activity of vascular NADPH oxide has been shown to restore eNOS functionality (Hink et al., 2001; Mollnau et al., 2002). Therefore, a correction of eNOS uncoupling combined with up-regulation of eNOS expression may be a promising therapeutic strategy for cardiovascular disease.

Zizyphi Spinosi semen (ZSS, Suanzaoren in Chinese) is the dried seed of Zizyphus jujuba Mill. var. spinosa (Bunge). ZSS has been widely used since antiquity in Traditional Chinese Medicine for the treatment of neurasthenia, anxiety, and insomnia. However, modern pharmacological studies revealed that this herb also has potent actions on the cardiovascular system, such as antiarrhythmic and blood pressure-lowering effects (Huang, 1999).

Here we report that betulinic acid from ZSS up-regulates eNOS and, at the same time, reduces the expression of NADPH oxidase in human endothelial cells. This results in enhanced bioactive NO. Therefore, compounds like betulinic acid may have therapeutic potential for cardiovascular diseases.

**Materials and Methods**

**Aqueous Extract of ZSS.** Ten-gram ZSS seeds were crushed, incubated with 100 ml of water for 30 min, and then boiled for another 30 min. The solution was collected using paper filter. After adding 100 ml of water, the solid phase was boiled again for 30 min and the mixture filtered with a paper filter. Filtrates from the both steps were pooled, mixed with an equal volume of ethanol, and placed in darkness at 4°C for 24 h. The precipitate was removed by centrifugation at 1000 g for 10 min. The supernatant was filtered using a 0.45-μm membrane. The solution was concentrated using a rotary evaporator at 40°C to reduce the volume down to 2 ml. This was considered a stock solution and used at different dilutions in the experiments.

**Determination of NO Synthesis Using an NO Analyzer.** EA.hy 926 cells were incubated with ZSS extract for 18 h, and total protein was isolated. Western blotting was performed using 50 μg of protein and a monoclonal anti-eNOS antibody (BD Biosciences PharMingen, Santa Clara, CA) for analyzing the mRNA expression levels of eNOS mRNA (Applied Biosystems; assay ID Hs00276431_m1 and Hs00609145_m1, respectively). mRNA expression levels of target genes were normalized to TATA box-binding protein (Applied Biosystems; assay ID Hs00457620_m1).

**Cell Culture.** Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion as described previously (Li et al., 1998). HUVEC-derived EA.hy 926 endothelial cells were kindly provided by Dr. Cora-Jean Edgell (Pathology Department, University of North Carolina, Chapel Hill, NC). EA.hy 926 endothelial cells were grown under 10% CO₂ in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1× HAT (hypoxanthine, aminopterin, and thymidine) (Invitrogen, Karlsruhe, Germany) (Li and Förstermann, 2000b).

**Analysis of eNOS Promoter Activity in Stably Transfected EA.hy 926 Cells.** A stable EA.hy 926 cell line was generated by transfection of EA.hy 926 cells with pGEl-uSNOS-Hu-3500-neo, which contains a neomycin-resistance gene and a 3.5-kb promoter fragment of human eNOS driving the luciferase reporter gene (Li et al., 1998). These EA.hy 926 cells were cultured in medium containing 1 mg/ml G418 compound. For analysis of eNOS promoter activity, the stably transfected cells were incubated with ZSS extract for 18 h. Cell lysates then were prepared, and luciferase activities were determined as described previously (Li et al., 1998). The luciferase activity, normalized for protein concentration of cell lysates, was used as a determinant of eNOS promoter activity.

**Real-Time RT-PCR for mRNA Expression Analyses.** In some experiments, eNOS mRNA expression was analyzed with quantitative real-time RT-PCR using an iCycler iq System (Bio-Rad Laboratories, Munich, Germany). Total RNA (500 ng) was used for real-time RT-PCR analysis with the QuantiTect Probe RT-PCR kit (QIAGEN, Hilden, Germany). Sequences of used eNOS primers were 5’-GTGG-GCTGTCCTGATGGACCCT-3’ (forward) and 5’-CCACAGGTGTCAGGTTGGC-3’ (reverse). The sequence of the dual-labeled TaqMan probe was 5’-AGTGGAGAATTCAAGTGCCCGTGGTCGCT-3’ (Li et al., 2004). TaqMan Gene Expression Assays (predesigned probe and primer sets) were obtained from Applied Biosystems (Foster City, CA) for analyzing the mRNA expression of Nox4 and p22phox (assay ID Hs00276431_m1 and Hs00609145_m1, respectively). mRNA expression levels of target genes were normalized to TATA box-binding protein (Applied Biosystems; assay ID Hs00457620_m1).

**Regulation of eNOS and NADPH Oxidase by Betulinic Acid**

This work was supported by the German Research Foundation (Li and Förstermann, 2000b).

**Author Contributions**

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Measurement of ROS Production by L-012 Chemiluminescence.

Production of ROS was determined using the luminol derivative L-012 [8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H) dione] (Li et al., 2006). In brief, EA.hy 926 cells were treated with 10 μM betulinic acid for 18 h in 96-well plates, and L-012-derived chemiluminescence was measured using a Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany) in Hanks' buffered salt solution (PAA Laboratories GmbH, Coelbe, Germany) containing 500 μM L-012. In additional experiments, cells were pretreated with 300 μM apocynin for 48 h before betulinic acid treatment. The photon counts were normalized for protein content of the cell lysates.

Statistics. Statistical differences between mean values were determined by analysis of variance followed by Fisher's protected least-significant-difference test for comparison of different means.

Results

Increased eNOS Promoter Activity, eNOS Expression, and NO Production in Response to a ZSS Extract.

EA.hy 926 cells stably transfected with a 3.5-kb human eNOS promoter fragment were treated with an aqueous extract of ZSS. This treatment resulted in a concentration-dependent increase in eNOS promoter activity (Fig. 1A). The maximal increase was seen with a 1:100 dilution of the aqueous ZSS extract (5 g of raw material per milliliter as stock solution). Treatment with the control solutions at any dilution did not have any effect on eNOS promoter activity. For further experiments, control solutions at a 1:100 dilution was used as control.

Treatment of naive EA.hy 926 cells with ZSS resulted in a concentration-dependent increase in eNOS mRNA expression, as analyzed with RNase protection assay (Fig. 1, B and C). Figure 1D demonstrates the time dependence of eNOS up-regulation in response to aqueous ZSS extract. A maximal effect was reached after 18 h. Therefore, this time frame was chosen for all other experiments. An 18-h treatment of EA.hy 926 cells with aqueous ZSS extract also increased eNOS protein (as analyzed with Western blot, Fig. 2, A and B). Finally, ZSS treatment for 18 h increased NO synthesis in EA.hy 926 cells in a concentration-dependent manner, as determined by the NO-ozone chemiluminescence assay (Fig. 2C).

Effects of ZSS Constituents on eNOS Expression in Human Endothelial Cells.

Jujuboside A and B had no effect on eNOS mRNA expression in human EA.hy 926 en-

Fig. 1. Extract of ZSS enhances eNOS promoter activity and eNOS mRNA expression. A, human EA.hy 926 endothelial cells were stably transfected with a 3.5-kb human eNOS promoter fragment driving a luciferase reporter gene. Cells were treated with the ZSS extract at the indicated dilutions (stock solution corresponding to 5 g/ml raw ZSS). Luciferase activity was analyzed as a determinant of eNOS promoter activity. B to D, human EA.hy 926 endothelial cells were treated for 18 h with ZSS extract, and eNOS mRNA expression was analyzed with RNase protection assays. B, an original gel (performed in triplicate). The gel shows the protected bands for eNOS and for β-actin (used for normalization). C and D, the concentration and time dependence of the effect of ZSS on eNOS mRNA expression, respectively. Symbols represent mean ± S.E.M., n = 9–15 (∗, p < 0.05; ∗∗, p < 0.01, ∗∗∗, p < 0.001, compared with control).

Fig. 2. ZSS extract increases eNOS protein expression and NO produc-

Fig. 3. ZSS extract induces eNOS protein expression and NO produc-

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dothelial cells (Fig. 3), and these compounds did not change eNOS promoter activity (up to 30 μM and an 18 h incubation; data not shown). Furthermore, betulin had no significant effect on eNOS mRNA expression (Fig. 4A). In contrast, an 18 h-treatment with betulinic acid significantly increased eNOS mRNA expression in both EA.hy 926 cells (Fig. 4A) and in HUVEC (Fig. 4B).

Increased eNOS Protein Expression and NO Production in Response to Betulinic Acid. Treatment of EA.hy 926 cells with 10 μM betulinic acid for 18 h resulted in a significant induction of eNOS protein, as determined by Western blot (Fig. 5A). The conditioned media from these cells showed higher potency in stimulating cGMP generation in RFL-6 reporter cells, indicating an increased NO production by EA.hy 926 cells (Fig. 5B).

Down-Regulation of Nox4 and p22phox Expression and Reduced ROS Production in Response to Betulinic Acid. Treatment with 10 μM betulinic acid for 18 h resulted in marked down-regulation of Nox4 and p22phox mRNA expression in EA.hy 926 cells (Fig. 6A) as well as in HUVEC (Fig. 6B). This effect seems to be independent of the eNOS-up-regulating effect of betulinic acid, because it was not prevented by the NOS inhibitor N(G)-nitro-L-arginine methyl ester (Fig. 7, L-NAME). In addition, treatment of EA.hy 926 cells with the NO donor spermine NONOate did not reduce Nox4 expression (Fig. 7). The reduced expression of NADPH oxidase subunits by betulinic acid was associated with a reduction of ROS production (Fig. 8). The effect of betulinic acid on ROS production was likely to be NADPH oxidase-dependent, because it was absent in the presence of apocynin, a NADPH oxidase inhibitor (Fig. 8).

Discussion
ZSS is one of the most commonly used Chinese herbs. It is a sedative and hypnotic drug with additional effect on the cardiovascular system (Huang, 1999).
ZSS protects cardiomyocytes from ischemic injury. Damage of cultured neonatal rat myocardial cells by deprivation of oxygen and glucose was markedly reduced by ZSS total saponins (Chen et al., 1990). Anoxia reoxygenation of cultured neonatal rat myocardial cells results in increased intracellular malondialdehyde and lipid peroxides, increased intercellular calcium concentration, and decreased SOD activity. All of these parameters were reversed by ZSS total saponins (Chen et al., 1990; Wan et al., 1997). In an animal model of endotoxin fever, the reduced SOD protein levels were prevented by treatment with ZSS (Peng et al., 1995). ZSS has also blood pressure-lowering effects. Intravenous injection of an aqueous solution of ZSS extract markedly decreased blood pressure in anesthetized rats, dogs, and cats without any significant effect on the coronary blood flow, heart rates, or myocardial contractility (Gu et al., 1987). Oral treatment of spontaneously hypertensive rats with ZSS jujubosides resulted in reduction of blood pressure (Zhang et al., 2003). Blood pressure reduction was observed as early as 30 min and lasted at least 3.5 h, and the effect declined after 7.5 h (Zhang et al., 2003). Moreover, treatment of hypercholesterolemic rabbits with ZSS for 3 months resulted in a reduction of total cholesterol, triglyceride, LDL, and atherosclerosis and an increase in HDL (Wu et al., 1989).

Molecular mechanisms underlying these cardiovascular effects are poorly understood. Interestingly, treatment of rats with ZSS resulted in increased plasma levels of NO by unknown mechanisms (Wang and Xie, 2004). Expression or activity of NOS isoforms was not analyzed in that study.

The present study is the first that systematically investigates the effect of ZSS and its constituents on the gene expression of eNOS. We found that ZSS increases eNOS promoter activity (Fig. 1A), eNOS mRNA (Fig. 1, B–D), and protein expression (Fig. 2, A and B), as well as NO production (Fig. 2C) in human endothelial cells.

The active constituents of ZSS include saponins, triterpenoids, flavonoids, alkaloids, and fatty acids (Huang, 1999; Li et al., 2005b; Zhao et al., 2006). The most important ZSS saponins are triterpenoid oligoglycosides, such as jujubosides A and B (Huang, 1999; Bai et al., 2003; Wang et al., 2005). The content of jujubosides A and B in ZSS is approximately 400 mg/kg (Wang et al., 2005). Triterpenoids found in ZSS include betulin and betulinic acid (Bai et al., 2003; Li et al., 2005b).

Jujuboside A and B showed no effect on eNOS promoter activity or eNOS mRNA expression (Fig. 3). Furthermore,
betulin had no effect on eNOS expression (Fig. 4A). Interestingly, treatment of human endothelial cells with betulinic acid resulted in a significant up-regulation of eNOS mRNA (Fig. 4) and protein (Fig. 5A) expression. The contents of betulinic acid and betulin in ZSS are approximately 7 and 3 mg/kg, respectively (Li et al., 2005b). In the present study, we used ZSS extract at a concentration of 5 g/ml. When cells were treated with this extract at a 1:100 dilution, the estimated final concentration of betulinic acid was in the low micromolar range. As shown in Fig. 4B, betulinic acid in increased eNOS mRNA expression even at 1 μM. Thus, betulinic acid is probably one of the compounds responsible for the eNOS up-regulation produced by ZSS.

Up-regulation of eNOS does not necessarily result in an increase in bioactive NO. Under pathological conditions of oxidative stress, eNOS is often uncoupled and dysfunctional (Forstermann and Munzel, 2006). In the vasculature, ROS can be produced by several enzyme systems, including enzymes of the respiratory chain, cytochrome P450 monooxygenases, uncoupled eNOS, xanthine oxidase, and NADPH oxidases. Although all of these enzymes contribute to the oxidative burden, evidence is accumulating that an initial generation of ROS by NADPH oxidases triggers the release of ROS by the other enzymes (Griendling, 2004). Moreover, NADPH oxidase-mediated oxidative stress is involved in the development and progression of atherosclerosis (Lassegue and Clempus, 2003). In diseased human coronary arteries, approximately 60% of vascular superoxide is derived from NADPH oxidases (Guzik et al., 2006).

The NADPH oxidase enzyme complex consists of two essential membrane-bound catalytic subunits, gp91phox/Nox2 and p22phox, which compose flavocytochrome b558, and several cytosolic regulatory components (Lassegue and Clempus, 2003). In the vasculature, the catalytic subunit gp91phox/Nox2 is a member of a family of homologous proteins termed Nox. In addition to gp91phox/Nox2, Nox1 and Nox4 are also expressed in blood vessels (Lassegue and Clempus, 2003; Griendling, 2004). Both Nox2 and Nox4 are found in endothelial cells (Griendling, 2004). Nox4 is probably the major Nox isofrom and the predominant ROS source in endothelial cells (Ago et al., 2004; Hu et al., 2005).

Interestingly, betulinic acid significantly reduced the expression of NADPH oxidase catalytic subunits Nox4 and p22phox (Fig. 6), which was associated with a reduction in ROS production (Fig. 8). Therefore, betulinic acid possesses a dual protective action on the vasculature. i) It up-regulates eNOS expression, thereby producing more eNOS protein, and ii) it down-regulates NADPH oxidase, thereby maintaining the up-regulated eNOS enzyme in a functional state.

In the past, we have found analogous combined effects of some other compounds. For example, the PKC inhibitor midostaurin up-regulates eNOS expression by a PKC-independent mechanism (Li and Forstermann, 2000b) and reduces vascular Nox1 expression by PKC inhibition (Li et al., 2006). By reducing NADPH oxidase-mediated oxidative stress, midostaurin reverses eNOS uncoupling in spontaneously hypertensive rats and in atherosclerosis-prone apoE-KO mice, which was associated with NO-mediated vasodilation and blood pressure reduction (Li et al., 2005a, 2006). In addition, red wine and its constituents (such as resveratrol, cinnamic acid, and hydroxycinnamic acid) up-regulate eNOS while preserving eNOS functionality (Wallerath et al., 2002, 2003, 2005). Direct antioxidant properties of these compounds, as well as the demonstrated down-regulation of Nox4 by resveratrol (H. Li, H. Xu, G. Spanier, and U. Forstermann, unpublished data), may contribute to this effect.

Thus, the combination of eNOS up-regulation and NADPH oxidase down-regulation may result in enhanced levels of bioactive NO and thus vascular protection. Indeed, treatment of endothelial cells with betulinic acid increased bioactive NO (that stimulated cGMP generation in RFL-6 reporter cells; Fig. 5B), indicating that the up-regulated eNOS by betulinic acid is maintained under a functional state.

The current study has certain limitations. i) The measurements of NO were indirect as nitrite/nitrate or stimulation of soluble guanylyl cyclase; however, at least the latter indicated that bioactive NO was increased by betulinic acid. ii) The main effects are shown on healthy cells that are not stressed. Further experiments are needed to find out whether betulinic acid can reverse eNOS uncoupling under conditions of oxidative stress. The suppression of NADPH oxidase expression by betulinic acid (Fig. 6, A and B) would favor such an action. (iii) Betulinic acid has been shown to have proapoptotic effects in cancer cells (Rzeski et al., 2006). We have confirmed this effect in the alveolar epithelial carcinoma cell line A549/8 (data not shown). However, in HUVEC, betulinic acid did not induce apoptosis. At concentrations of 1 and 10 μM, betulinic acid even reduced caspase 3/7 activity in HUVEC (data not shown).

In conclusion, the present study demonstrates that betulinic acid from the Chinese herb ZSS up-regulates eNOS expression and down-regulates NADPH oxidase expression in human endothelial cells. This results in an increased amount of functional eNOS protein, enhanced production of bioactive NO, and thus vasoprotection (even in disease conditions with increased oxidative stress).

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