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Effects of Vasoactive Chinese Herbs on the Endothelial NO System

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1. Introduction

In the vasculature, nitric oxide (NO) is produced from the endothelium mainly by endothelial NO synthase (eNOS), which is activated by agonists such as bradykinin and acetylcholine or by shear stress produced by the flowing blood. NO is a potent vasodilator and protects blood vessels from thrombosis by inhibiting platelet aggregation and adhesion. In addition, endothelial NO possesses multiple anti-atherosclerotic properties, which include (i) prevention of leukocyte adhesion to the vascular endothelium and leukocyte migration into the vascular wall; (ii) decreased endothelial permeability, reduced influx of lipoproteins into the vascular wall and inhibition of low density lipoprotein (LDL) oxidation; and (iii) inhibition of DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells [1, 2]. Recent studies suggest that eNOS is also involved in mitochondrial biogenesis, anti-aging effects and extension of lifespan in mammals [3, 4].

Based on the abovementioned protective effects of eNOS-derived NO, a pharmacological enhancement of NO production is of therapeutic interest. Indeed, numerous Chinese medicinal plants, herbal preparations, or isolated compounds thereof have been shown to stimulate endothelial NO production, which is likely to be a contributing mechanism for their therapeutic effects.

2. Regulation of endothelial NO production

Endothelial NO production is regulated at different levels, including

- Regulation of eNOS expression: expression of the eNOS enzyme is regulated at both the transcriptional and post-transcriptional levels. Estrogens, for example, increase eNOS expression by stimulating eNOS promoter activity. TNF α , on the other hand, reduces eNOS expression by destabilizing eNOS mRNA [5, 6]. Several Chinese herbal products have been shown to enhance eNOS expression (Table 1).
- Regulation of eNOS activity by post-translational modifications: the enzymatic activity of eNOS is regulated by different cellular events such as increased intracellular Ca²⁺, interactions with substrates, co-factors, adaptors and regulatory proteins, and through shuttling between distinct subcellular domains [7]. In addition, eNOS activity is also regulated by post-translational modification of the eNOS protein. For example, phosphorylation of serine 1177 enhances eNOS activity, whereas phosphorylation of

threonine 495 decreases eNOS activity [7]. Recent studies indicate that eNOS activity can be also enhanced by SIRT1-mediated deacetylation of lysine residues in the calmodulin-binding domain [8].

- Regulation of eNOS activity by changing the intracellular concentration of asymmetric dimethylarginine (ADMA): elevated plasma levels of ADMA have been shown to be associated with cardiovascular events and mortality. ADMA is believed to be an endogenous eNOS inhibitor, although NO-independent effects of ADMA have also been reported [9]. ADMA is formed during proteolysis and is degraded by the intracellular enzyme dimethylarginine dimethylaminohydrolase (DDAH). Decreased DDAH expression/activity is evident in disease states associated with endothelial dysfunction [10].
- Regulation of eNOS functionality: under a number of pathological conditions, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in production of superoxide rather than NO. This phenomenon is referred to as “eNOS uncoupling” [1, 5]. A number of potential mechanisms have been reported to contribute to eNOS uncoupling. Among all of these mechanisms, a deficiency of the NOS cofactor tetrahydrobiopterin (BH₄) seems to be the primary cause for eNOS uncoupling in pathophysiology. In BH₄ deficiency, the reduction of molecular oxygen still occurs at the heme site of eNOS, but oxidation of the guanidine nitrogen of L-arginine is prevented, so that the reduced oxygen comes off the enzyme as superoxide [11, 12]. eNOS-mediated superoxide production has been observed in animal models of atherosclerosis, hypertension, diabetes mellitus and nitroglycerin tolerance, and also in patients with endothelial dysfunction resulting from hypercholesterolemia, diabetes mellitus, or essential hypertension, in chronic smokers, and in nitroglycerin-treated patients [11, 12]. Thus, eNOS uncoupling turns eNOS from a NO-producing protective enzyme into a superoxide-generating deleterious molecule. It is therefore of therapeutic interest to reverse eNOS uncoupling and restore eNOS functionality. An elevation of endothelial BH₄ levels (by enhancing BH₄ biosynthesis and/or by preventing oxidative stress-mediated BH₄ oxidation) may reverse eNOS uncoupling [13]. Chinese herbs containing large amounts of polyphenolic compounds with antioxidant properties may have the potential to prevent BH₄ oxidation and eNOS uncoupling.
- Regulation of NO bioactivity by reducing the levels of reactive oxygen species (ROS): NO can be rapidly inactivated by superoxide. A reduction of oxidative stress (by downregulating ROS-producing enzymes, upregulating antioxidant enzymes, or by ROS scavenging activities) may enhance NO bioactivity by two means: prevention of eNOS uncoupling and reduction of superoxide-mediated NO inactivation [1].

3. Searching for eNOS-enhancing Chinese herbs

Numerous Chinese herbs have been shown to enhance endothelial NO production. These reports are summarized in Table 1. This chapter focuses on our own findings.

EA.hy 926 cells are an immortalized endothelial cell line derived from human umbilical vein endothelial cells (HUVEC). This cell line has been generated by fusing HUVEC with the permanent human alveolar epithelial cell line A549 [14]. We cloned the 5'-flanking region (3.5 kb in length) of the human eNOS gene into pGL₃-neo, which contains a promoterless

luciferase reporter gene and a neomycin resistance gene [15]. Stable transfection of EA.hy 926 cells with this construct (selection using G418) resulted in an immortalized human endothelial cell line (termed “stable EA.hy cells” for simplicity) that expresses the luciferase gene driven by the human eNOS promoter. Luciferase activity in cell homogenates, which can be easily measured in a luminometer or on a chemiluminescence plate reader, is used as a determinant of promoter activity of the human eNOS gene.

To search for eNOS-regulating Chinese herbs, we generated aqueous extracts of 17 herbs possessing “circulation-improving” effects according to traditional Chinese medicine (TCM) [16]. These were:

- *Angelicae sinensis radix*
- *Astragali radix*
- *Carthami flos*
- *Celosiae semen*
- *Chrysanthemi indicis flos*
- *Eucommiae cortex*
- *Ligustici radix*
- *Metaphyreum roseum*
- *Moutan radice cortex*
- *Paeoniae rubrae radix*
- *Panacis notoginseng radix*
- *Persicae semen*
- *Prunella vulgaris L.*
- *Puerariae radix*
- *Salviae miltiorrhizae radix*
- *Uncariae ramulus et unci*
- *Zizyphi spinosae semen*

Human EA.hy 926 endothelial cells stably transfected with a 3.5 kb fragment of the human eNOS promoter (stable EA.hy cells) were treated with each extract (corresponding to 5 g of raw plant extract per ml) at dilutions of 1:100000 to 1:300 for 18 hours to study their effects on eNOS promoter activity. Ten herbal extracts increased eNOS promoter activity in a concentration-dependent manner with a maximal effect over 150%: *Carthami flos*, *Chrysanthemi indicis flos*, *Eucommiae cortex*, *Ligustici radix*, *Paeoniae rubrae radix*, *Prunella vulgaris L.*, *Puerariae radix*, *Uncariae ramulus et unci*, *Salviae miltiorrhizae radix* and *Zizyphi spinosae semen*.

These ten herbal extracts were further analyzed in a second screening (RNase protection assay) for their effect on eNOS mRNA levels in normal EA.hy 926 cells. In this experiment, only *Prunella vulgaris L.*, *Salviae miltiorrhizae radix* and *Zizyphi spinosae semen* significantly increased eNOS mRNA expression [16].

Our screening procedure using stable EA.hy cells has some limitations: (i) it is based on eNOS promoter activity and the positive results must be verified by additional methods for analyses of mRNA expression to exclude false positive hits. (ii) Herbs/compounds that regulate NO production via mechanisms other than modulating eNOS expression will provide false negative results. For example, *Astragali radix* had no effect on eNOS expression and was negative in our screening. However, *Astragali radix* can stimulate NO production from eNOS by enhancing eNOS enzymatic activity [17]. Despite of its limitations, this screening procedure has the advantages of being a low-cost method of relatively high

efficiency which is easy to perform. Because of these strengths, it has also been used by pharmaceutical companies in the screening for eNOS enhancers [18]. Based on this screening, we have identified *Prunella vulgaris L.*, *Salviae miltiorrhizae radix* and *Zizyphi spinosae semen* as eNOS-regulating Chinese herbs.

Plant	Compounds / extracts	Cells / models	Effects on eNOS	Additional effects	References
Astragali radix	extract	HUVEC	eNOS activity↑	VEGF↑, PI3K-Akt↑, angiogenesis↑	[17]
Epimedii herba	icariin	coronary artery isolated from canine	eNOS activity↑	vasodilation	[74]
Ginseng	ginsenosides	I/R in Langendorff hearts; HAEC	eNOS-P↑	cardiac function↑, coronary perfusion flow↑, prostacyclin↑, PI3K-Akt↑	[75]
Ginseng (Korean red ginseng)	saponin fraction	Hypertensive rats	eNOS activity↑	blood pressure↓	[76]
Magnolia officinalis	honokiol	oxLDL-treated HUVEC	eNOS protein expression↑	adhesion molecules↓, EC-monocyte interaction↓, LDL-oxidation↓, ROS↓	[77]
Panax notoginseng	saponin extract	HUVEC / zebrafish	eNOS-P↑	angiogenesis↑, VEGF↑, PI3K-Akt↑	[78]
Polygonum multiflorum	TSG	aorta isolated from fat-fed rats	eNOS expression↑	endothelial function↑, intimal remodeling↓, iNOS↓	[79]
Pueraria lobata	puerarin	fat-fed rats	eNOS expression↑	cholesterol↓	[80]
Prunella vulgaris	extract	EA.hy / HUVEC	eNOS expression↑	NO↑	[16]
Salvia miltiorrhiza (Danshen)	crypto-tanshinone	HUVEC	eNOS expression↑	ET-1↓, NF-κB↓	[81]
	magnesium lithospermate B	HUVEC (hyperglycemia) / OLETF rats	eNOS-P↑	Endothelial function↑, Akt↑, Nrf-2↑, HO-1↑	[82]
	tanshinone IIA	angiotensin II-treated rat cardiac fibroblasts	eNOS-P↑	ET-1↓, ROS↓, fibroblast proliferation ↓	[83]
	tanshinone IIA	renovascular hypertension model in hamsters	eNOS expression↑ eNOS -P↑	Vasodilation, blood pressure↓	[84]
	extract / ursolic acid	EA.hy 926 / HUVEC	eNOS expression↑	NADPH oxidase↓	[24]
	extract	rat hypoxic pulmonary hypertension	eNOS expression↑	media thickening↓, iNOS↓	[85]
Seabuckthorn	flavonoids	EA.hy 926	eNOS expression↑	LOX-1↓, cell death↓	[86]

Plant	Compounds / extracts	Cells / models	Effects on eNOS	Additional effects	References
Sophora flavescens roots	matrine	RIMEC	eNOS expression↑	IL-6, IL-8 & sICAM-1↓	[87]
Szechwan lovage rhizome	TMP	Cerebral vasospasm in rabbit	eNOS expression↑	cerebral vasospasm↓	[88]
Xanthoceras sorbifolia	ethanol extract	Rat aorta	eNOS activity↑	vasodilation	[89]
Zizyphi Spinosi semen	betulinic acid	EA.hy / HUVEC	eNOS expression↑	ROS↓	[62]
Combinations & compounds					
A&A	decoction	obstructed rat kidney	eNOS activity↑	renal fibrosis↓, ROS↓	[90]
ELCAS	extract	H ₂ O ₂ -treated ECV304	eNOS expression↑	SOD↑, CAT↑, GPx↑	[91]
Qing Huo Yi Hao	TMP	glucose-treated bEnd.3	eNOS-P↑	ROS↓, Akt↑, UCP2↑	[92]
Refined Qing Kai Ling	injection	MCAO in rats	eNOS expression↑	infarct size↓	[93]
Shen-fu	injection	myocardial I/R in diabetic rats	eNOS-P↑	infarct size↓, Akt↑	[94]
Tongxinluo	compound	HUVEC / aortic rings from rats on a methionine-rich diet	eNOS expression↑	Endothelial function↑, PI3K- Akt↑	[95]
		myocardial no-reflow and I/R in minipigs	eNOS-P↑	PKA↑, infarct size↓	[96]

A&A, a combination of roots *Astragalus membranaceus* var. *mongholicus* and *Angelica sinensis*; bEnd.3, mouse brain microvascular cells; CAT, catalase; EA.hy 926, an immortalized human endothelial cell line derived from HUVEC; ECV 304, a misidentified (as endothelial cells) human bladder cell line of epithelial origin; ELCAS, an extract of *Ligusticum chuanxiong* and *Angelica sinensis*; eNOS-P, eNOS phosphorylation at serine 1177; GPx, glutathione peroxidase; HAEC, human aortic endothelial cells; HO-1, heme oxygenase-1; H/R, hypoxia/reoxygenation; HUVEC, human umbilical vein endothelial cells; I/R, ischemia-reperfusion injury; MCAO, middle cerebral artery occlusion; Nrf-2, nuclear factor erythroid 2-related factor-2; OLETF, Otsuka Long-Evans Tokushima Fatty rats; RIMEC, rat intestinal microvascular endothelial cells; ROS, reactive oxygen species; SOD, superoxide dismutase; TMP, tetramethylpyrazine; TSG, 2,3,4,5-tetrahydroxystilbene 2-O-beta-D-glucoside; UCP2, uncoupling protein 2.

Table 1. Effects of Chinese herbs on endothelial NO production.

3.1 *Prunella vulgaris* L. (PVL)

PVL is used in TCM as well as in Western herbal medicine. In the West, the plant has been used primarily as a remedy to alleviate pains in the throat, to treat fevers and to accelerate wound healing. Modern pharmacological studies have revealed a wide array of biological effects and numerous therapeutic possibilities for the herb, including anti-viral and anti-bacterial effects, immunomodulatory, anti-allergy and anti-cancer potential, as well as antioxidant activity [16].

In TCM, PVL (fruiting spikes) is used as an anti-microbial, anti-inflammatory and anti-tumor drug [19], but it is also commonly used as a component in combination therapy for hypertension.

PVL extracts relax isolated epinephrine-precontracted rabbit aorta [20]. Intravenous injection of PVL saponins results in a reduction of both systolic and diastolic blood pressures in anesthetized rats [21]. A PVL-containing Chinese herb combination (consisting of *Crataegus pinnatifida* Bge, *Uncariae ramulus et uncis*, *Alisma orientalis radix* and PVL at 1:1:1:1, w/w) reduces blood pressure and lowers cholesterol and triglyceride in hypertensive and hypercholesterolemic patients [22].

Our study demonstrated that PVL is an effective eNOS-upregulating herb; it significantly increases eNOS promoter activity, eNOS mRNA and protein expression and NO production in human endothelial cells [16].

PVL extracts contain a variety of chemical constituents, including triterpenoids (such as ursolic acid, betulinic acid, oleanolic acid, vulgarsaponins), steroids (such as β -sitosterol, stigmasterol, α -spinasterol), flavonoids (such as rosmarinic acid, luteolin, cynaroside, homoorientin, quercetin), coumarins (such as umbelliferone, scopoletin, esculetin), organic acids (such as caffeic acid, palmitic acid, stearic acid, oleic acid, arachidic acid, lauric acid, myristic acid), sugars, as well as essential oils [23].

Importantly, our studies have demonstrated that ursolic acid (also present in *Salviae miltiorrhizae radix*) [24], betulinic acid (also a constituent of *Zizyphi spinosae semen*) [24], luteolin and cynaroside (also constituents of artichoke, *Cynara scolymus* L.) [25] are eNOS-upregulating compounds. Ursolic acid and betulinic acid are two of the main PVL triterpenoids; luteolin and cynaroside are two of the main PVL flavonoids [23]. Therefore, these four compounds, possibly in combination with other yet unidentified compounds, may be responsible for the observed eNOS-upregulating effect of PVL.

Our recent experience indicates that the effects of PVL on eNOS may vary significantly from batch to batch. With the last two batches of PVL products we bought recently, we could not find any eNOS-enhancing activities. This might be due to concentration variation of the active constituents in the PVL plant.

3.2 *Salviae miltiorrhizae radix* (Danshen)

Danshen, the dried root of *Salvia miltiorrhiza* Bunge (Lamiaceae), is one of the most commonly used TCM remedies. A Danshen-containing preparation was the first TCM product approved for phase II and III clinical trials by the Food and Drug Administration (FDA) [26]. Ancient TCM books describe Danshen as a drug “improving circulation” and “removing blood stasis”. Today, Danshen is available in China, Japan, the United States, and also in many European countries and is used for the treatment of angina pectoris, hyperlipidemia, and acute ischemic stroke [26]. Clinical trials have indicated that Danshen preparations are superior to nitroglycerin or isosorbide dinitrate for the treatment of stable angina pectoris, with respect to efficacy and side effects [27, 28].

Various *in vitro* and *in vivo* studies have demonstrated that several constituents of Danshen can improve microcirculation, dilate coronary arteries, increase blood flow, and prevent myocardial ischemia. Aqueous Danshen extracts and purified active principles of Danshen (tanshinones) have been shown to cause vasodilation of coronary, renal, femoral, and mesenteric arteries, and suppress systemic blood pressure in rats and rabbits [29, 30]. In a rat acute myocardial infarction model, Danshen extracts increased the survival rate and

reduced the infarct size to an extent comparable with that of the angiotensin converting enzyme inhibitor ramipril [31]. Tanshinone IIA, a pharmacologically active component isolated from Danshen, reduced myocardial infarct size by about 50% in a rabbit ischemia-reperfusion model [32].

Interestingly, the cardiovascular protective effects of Danshen resemble the action profile of endothelium-derived NO. Danshen extracts and constituents inhibit platelet aggregation [33] and attenuate neutrophil-endothelial adhesion [34]. Danshen also lowers plasma cholesterol levels, enhances smooth muscle apoptosis and attenuates neointimal hyperplasia in the balloon-injured abdominal aorta of hypercholesterolemic rabbits [35]. Danshen has also been shown to reduce lipid peroxidation, inhibit LDL oxidation, and reduce atherosclerosis in cholesterol-fed rabbits [36].

In stable EA.hy cells we have identified Danshen as an eNOS-enhancer. Danshen extracts increase eNOS promoter activity, eNOS mRNA and protein expression, as well as endothelial NO production [24]. Danshen extracts contain large amounts of polyphenolic compounds with antioxidant properties [26, 37]. This may prevent BH₄ oxidation and eNOS uncoupling.

So far, more than 100 compounds have been isolated and identified from Danshen. Most of the lipophilic compounds are diterpene chinone compounds of the tanshinone type, including tanshinone I, IIA, and IIB, cryptotanshinone, dihydrotanshinone, and other related compounds [26, 37]. Hydrophilic constituents include polyphenolic acids (such as various salvianolic acids) and related compounds (such as danshensu, i.e. salvianic acid A, protocatechuic aldehyde, and protocatechuic acid); but also rosmarinic acid and isoferulic acid. Baicalin and ursolic acid have been isolated from alcohol extracts of Danshen [26].

In EA.hy 926 cells, an aqueous extract of Danshen and a methanol extract of the plant increase eNOS promoter activity, eNOS mRNA and protein expression. On the contrary, a dichloromethane extract does not change eNOS gene expression [24]. Thus, hydrophilic and alcohol-soluble, but not lipophilic constituents of Danshen seem to be responsible for its eNOS-upregulating effect. Accordingly, the commercially available lipophilic compounds (tanshinone I, tanshinone IIA, cryptotanshinone and dihydrotanshinone) of Danshen have no effect on eNOS expression in EA.hy 926 cells [24]. We have also tested several commercially available hydrophilic compounds, including protocatechuic acid, protocatechuic aldehyde, rosmarinic acid, isoferulic acid, salvianic acid A, and salvianolic acid B. They show no significant effects on eNOS expression [24]. Therefore, the hydrophilic compounds that are responsible for the eNOS-upregulating effect of Danshen still remain to be identified. Among the alcohol-soluble compounds, we have found that ursolic acid, but not baicalin, significantly enhances eNOS mRNA and protein expression [24]. Therefore, ursolic acid is likely to represent one of the compounds responsible for enhanced eNOS expression in response to Danshen.

3.3 Ursolic acid

Ursolic acid is a secondary plant metabolite not only found in Danshen [38], but also widespread in some other plants including apple pomace, rosemary leaves, and sage leaves (*Salvia officinalis*) [39]. This pentacyclic triterpenoid has emerged as a multifunctional compound with diverse pharmacological properties, including anticancer, antioxidant, anti-inflammatory, anti-HIV, antimicrobial, and hepatoprotective activities. Currently, ursolic acid is in human clinical trials for treating cancer and skin wrinkles [40, 41]. In addition, ursolic acid possesses anti-obestic and anti-diabetic effects. It prevents abdominal adiposity

[42, 43], improves pancreatic beta-cell function [44], ameliorates glucose intolerance [45], inhibits hepatic glucose production in diabetic mice [46], and inhibits diabetic nephropathy [47, 48].

Also in the cardiovascular system, ursolic acid shows therapeutic effects. In rat models of hypertension, ursolic acid prevents the development of severe hypertension which may be attributed to a potent diuretic/saluretic activity and a negative chronotropic effect. In addition, ursolic acid shows antihyperlipidemic (reduction of LDL and triglycerides), antioxidant (upregulation of glutathione peroxidase, GPx, and superoxide dismutase, SOD), and hypoglycemic effects [49].

The reported effects of ursolic acid on atherogenesis are controversial. TNF α -induced E-selectin expression is shown to be suppressed by ursolic acid via the inhibition of NF- κ B [50]. In the rat carotid artery injury model, ursolic acid has been demonstrated to inhibit neointima formation [51]. In contrast, a recent study has shown that oral treatment of apolipoprotein E-knockout mice with ursolic acid for 24 weeks accelerates atherosclerotic plaque formation in a dose-dependent manner [52]. In the latter study, ursolic acid inhibited endothelial proliferation and induced endothelial cell death. Ursolic acid caused DNA damage, followed by the activation of a p53-, BAK-, and caspase-dependent cell-death pathway [52]. Further studies are needed to clarify this controversy.

We have provided the first evidence that ursolic acid enhances eNOS mRNA and protein expression in human endothelial cells [24]. This leads to increased NO production and improved endothelial cell function. In a recent study by Lee et al., treatment of human coronary artery endothelial cells with ursolic acid increased tube formation, endothelial cell migration capacities and the expression of allograft inflammatory factor-1 (AIF-1, a mediator of vasculogenesis) through an NO-related mechanism [53]. In a mouse hind limb ischemia model, ursolic acid enhanced eNOS and AIF-1 expression, and increased collateral blood flow and capillary density through the induction of neovascularization [53].

In addition to this stimulatory effect on gene expression observed at concentrations of 1-10 μ M, ursolic acid at higher concentrations also stimulated eNOS activity. In organ chamber experiments with isolated rat aorta, ursolic acid (and a methanolic extract of *Lepchinia caulescens*) induced an endothelium-dependent, NO-mediated vasodilation (EC₅₀ for ursolic acid 44 μ M) [54].

Ursolic acid also possesses anti-oxidative effects. It has been shown to reduce endothelial superoxide production by suppressing the expression of NOX4 [24], which is the predominant NADPH oxidase isoform in endothelial cells [55]. In addition, ursolic acid also enhances ROS inactivation by upregulating the expression/activity of antioxidant enzymes, e.g., GPx and SOD [49]. Thus, ursolic acid may also have the potential to prevent BH₄ oxidation and eNOS uncoupling.

3.4 Zizyphi spinosae semen (ZSS)

ZSS is a sedative and hypnotic drug with additional effects on the cardiovascular system [19]. ZSS protects cardiomyocytes from ischemic injury, and oxygen and glucose deprivation-induced damage of cultured neonatal rat myocardial cells can be markedly reduced by ZSS total saponins [56]. 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 can be reversed by ZSS total saponins [56, 57].

ZSS has also antihypertensive effects. Intravenous injection of an aqueous solution of ZSS extract markedly decreases blood pressure in anesthetized rats, dogs, and cats without any significant effect on coronary blood flow, heart rate, or myocardial contractility [58]. Oral treatment of spontaneously hypertensive rats with ZSS jujubosides results in a reduction in blood pressure [59]. Blood pressure reduction can be observed as early as 30 min and lasted for at least 3.5 h; the effect declined after 7.5 h [59]. Moreover, treatment of hypercholesterolemic rabbits with ZSS for three months leads to a reduction in total cholesterol, LDL cholesterol and triglycerides, an increase in HDL and a decrease in atherosclerotic lesions [60].

The molecular mechanisms underlying these cardiovascular effects are poorly understood. Interestingly, treatment of rats with ZSS resulted in increased plasma levels of NO through unknown mechanisms [61]. We have found that ZSS increases eNOS promoter activity, eNOS mRNA and protein expression, as well as NO production in human endothelial cells [62].

The active constituents of ZSS include saponins, triterpenoids, flavonoids, alkaloids, and fatty acids [19, 63, 64]. The most important ZSS saponins are triterpenoid oligoglycosides, such as jujubosides A and B. Triterpenoids found in ZSS include betulin and betulinic acid [63, 65].

Jujuboside A, B and betulin show no effect on eNOS promoter activity or eNOS mRNA expression. Interestingly, treatment of human endothelial cells with betulinic acid results in a significant up-regulation of eNOS mRNA and protein expression [62]. The content of betulinic acid in ZSS is approximately 7 mg/kg [63]. When cells are treated with this ZSS extract (concentration of 5 g/ml) at a 1:100 dilution, the estimated final concentration of betulinic acid is in the low micromolar range. In our study, betulinic acid increases eNOS mRNA expression even at 1 μ M. Thus, betulinic acid is likely to be one of the compounds responsible for the eNOS up-regulation induced by ZSS [62].

3.5 Betulinic acid

The pentacyclic triterpenoid betulinic acid is not only found in ZSS, but is also widespread in fruit peel, leaves and stem bark of several species of plants, including white birch bark (*Betula pubescens*), plane bark (*Plantanus acerifolia*), rosemary leaves (*Rosmarinus officinalis*), Ber tree (*Ziziphus mauritiana*) and selfheal (*Prunella vulgaris*) [39, 66]. The compound is mainly known for its anti-tumor, anti-viral and anti-inflammatory activities [66, 67]. Our study has identified betulinic acid as an eNOS-stimulating compound [62].

Interestingly, accumulating data in the recent years support a protective effect of betulinic acid in the cardiovascular system. In a rat renal ischemia/reperfusion (I/R) injury model, betulinic acid attenuates I/R-induced oxidant responses, inhibits microscopic damage, and improves renal function by regulating the apoptotic function of leukocytes and inhibiting neutrophil infiltration [68]. Recently, betulinic acid and ursolic acid have been identified as selective agonists of the G protein-coupled receptor TGR5, which plays an important role in the control of energy metabolism. This suggests the therapeutic potential of betulinic acid and ursolic acid for metabolic diseases [69].

In HUVEC, betulinic acid has been shown to inhibit TNF α -induced ROS production and NF- κ B activation. The resulting inhibition of endothelial activation and leukocyte adhesion points to a protective role of the compound against vascular inflammation [70].

We have found that betulinic acid upregulates eNOS expression in endothelial cells [62]. As mentioned above, upregulation of eNOS does not necessarily result in an increase in bioactive NO. Under pathological conditions of oxidative stress, eNOS is often uncoupled and dysfunctional. The primary cause of eNOS uncoupling is a deficiency of its cofactor BH₄ due to oxidative stress-mediated oxidation (e.g. by peroxynitrite) [1, 12]. Importantly, betulinic acid also reduces the expression of NADPH oxidases (NOX4 and p22phox) [62], a major source of ROS in the vasculature [71]. We have demonstrated that betulinic acid reduces the levels of peroxynitrite in the mouse *in vivo* [72]. These results suggest the potential of betulinic acid to reverse eNOS uncoupling. In a mouse stroke model, betulinic acid upregulates eNOS and downregulated NADPH oxidases, events which are associated with a reduction in infarct size [72].

Our recent results indicate that betulinic acid not only enhances eNOS expression but also enhances eNOS enzymatic activity. Treatment of human endothelial cells with betulinic acid leads to phosphorylation of eNOS at serine 1177 and dephosphorylation of eNOS at threonine 495, and an increase in NO production [Hohmann N, Xia N, Forstermann U and Li H, unpublished data]. This is consistent with a recent report demonstrating that betulinic acid induces an endothelium-dependent, NO-mediated relaxation of isolated rat aorta [73].

4. Conclusion

Numerous vasoactive Chinese herbs possess stimulating effects on endothelial NO production, e.g. by enhancing eNOS expression and/or by modulating eNOS phosphorylation status. Such molecular events are likely to be contributing mechanisms for the therapeutic effects of these herbs described in traditional Chinese medicine.

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During the recent years, traditional Chinese medicine (TCM) has attracted the attention of researchers all over the world. It is looked upon not only as a bright pearl, but also a treasure house of ancient Chinese culture. Nowadays, TCM has become a subject area with high potential and the possibility for original innovation. This book titled *Recent Advances in Theories and Practice of Chinese Medicine* provides an authoritative and cutting-edge insight into TCM research, including its basic theories, diagnostic approach, current clinical applications, latest advances, and more. It discusses many often neglected important issues, such as the theory of TCM property, and how to carry out TCM research in the direction of TCM property theory using modern scientific technology. The authors of this book comprise an international group of recognized researchers who possess abundant clinical knowledge and research background due to their years of practicing TCM. Hopefully, this book will help our readers gain a deeper understanding of the unique characteristics of Chinese medicine.

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