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ORIGINAL PAPER

Pterostilbene, an Active Constituent of Blueberries, Stimulates Nitric Oxide Production via Activation of Endothelial Nitric Oxide Synthase in Human Umbilical Vein Endothelial Cells

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Abstract Endothelial dysfunction, a key process in development of cardiovascular diseases, is largely due to reduced nitric oxide (NO) derived from endothelial NO synthase (eNOS). Resveratrol has been reported to stimulate NO production via estrogen receptor α (ERα) activation in endothelial cells. Here, we investigated whether two natural methylated analogs of resveratrol, pterostilbene (Pts) and *trans*-3,5,4'-trimethoxystilbene (TMS), similarly to resveratrol, could influence endothelial NO release in human umbilical vein endothelial cells (HUVECs). In HUVECs exposed to Pts or TMS, NO production and phosphorylation of eNOS, protein kinase B (Akt), and ERα were measured by using a fluorimetric NO assay kit and Western blot analysis, respectively. Dimethylated Pts, but not trimethylated TMS, stimulated dose-dependent NO production via eNOS phosphorylation. Pts also stimulated dose-dependent phosphorylation of Akt, but not of ERα. NO production and eNOS phosphorylation in response to Pts were significantly abolished by the phosphoinositide 3-kinase (PI3K)/Akt inhibitor LY294002, but not by the ERα antagonist ICI182780. Our results suggest that Pts, but not TMS, is capable of inducing eNOS phosphorylation and the subsequent NO release, presumably, by activating PI3K/Akt pathway. The potential efficacy of Pts, an active constituent of blueberries, may aid in the prevention of cardiovascular diseases characterized by endothelial dysfunction.

Keywords Pterostilbene · NO · eNOS · Endothelial cells · Endothelial dysfunction

Abbreviations

Akt	Protein kinase B
DMSO	Dimethyl sulfoxide
eNOS	Endothelial nitric oxide synthase
ERα	Estrogen receptor α
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
L-NAME	N^G-Nitro-*L*-arginine methyl ester
MTT	3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NO	Nitric oxide
PI3K	Phosphoinositide 3-kinase
Pts	Pterostilbene
TMS	*trans*-3,5,4'-Trimethoxystilbene

Introduction

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene; Pts) is a natural dietary compound predominantly found in blueberries [1, 2]. It is a natural dimethylated analog of resveratrol (*trans*-3,5,4'-trihydroxystilbene) that shows health benefits, including cardioprotective activities [3, 4]. Because of their structural similarity, Pts and resveratrol have very similar pharmacologic properties [5]; however, due to the presence of two methoxyl groups, Pts has increased bioavailability in comparison to resveratrol [5]. Indeed, Pts has been demonstrated to have 80 % bioavailability compared to 20 % for

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resveratrol in animal studies [5]. Another natural trimethylated analog of resveratrol, *trans*-3,5,4'-trimethoxystilbene (TMS), has greater plasma exposure, a longer half-life and lower clearance rates in rats [6].

Endothelial dysfunction has been implicated not only in many cardiovascular diseases but also in the pathogenesis of atherosclerosis [7]. Endothelial dysfunction is characterized by reduction of the bioavailability of vasodilators, particularly endothelial nitric oxide (NO), and/or an increase in endothelium-derived contracting factors [8, 9]. NO, which is synthesized endogenously by NO synthase (NOS), has a wide range of biological properties [10]. In endothelial cells, NO is produced mainly by endothelial NOS (eNOS), of which activity is regulated not only by increased intracellular Ca^{2+} levels but also by its phosphorylation. At least six eNOS phosphorylation sites have been identified so far; however, major changes in eNOS function have been reported for the phosphorylation of serine 1177 (for activation) and threonine 495 (for inhibition) in the human eNOS sequence [11]. The eNOS phosphorylation has been shown to be regulated by a complex series of regulatory mechanisms. A number of kinases have been reported to phosphorylate eNOS and increase NO production. Some of them, such as protein kinase A, are activated by an increase in intracellular Ca^{2+}, whereas kinases, such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), can be activated independently of an increase in intracellular Ca^{2+} [12, 13].

There is a growing body of evidence that some naturally occurring compounds derived from dietary sources or from specific medicinal plants may positively influence endothelial NO production and, thus, promote endothelial function [7]. The identification of such compounds and better understanding of their molecular mechanism of action may provide valuable strategies for the prevention of cardiovascular diseases. It has been previously reported that the naturally occurring resveratrol is capable of stimulating NO production through its activation of estrogen receptor α (ERα) in endothelial cells [14]; and this prompted us to examine whether Pts, a dimethylated analog of resveratrol, and TMS, a trimethylated analog of resveratrol, could also stimulate endothelial NO production. Thus, the goal of the experiments reported in this paper was to examine whether two methylated analogs of resveratrol, Pts and TMS, could influence eNOS phosphorylation and NO release in cultured human umbilical vein endothelial cells (HUVECs).

Materials and Methods

Materials

Pts, 17β-estradiol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin, *L*-arginine, Hank's balanced salt solution (HBSS), luteolin, combretastatin A4, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TMS was obtained from Cayman Chemical (Ann Arbor, MI, USA). LY294002 and N^G-nitro-*L*-arginine methyl ester (L-NAME) were purchased from Calbiochem (La Jolla, CA, USA). ICI182780 was obtained from Tocris Biosciences (Ellisville, MO, USA). The horseradish peroxidase (HRP)-conjugated second antibodies to rabbit and mouse IgG and the primary antibodies to phosphor (p)-ERα, ERα, p-eNOS, eNOS, p-Akt and Akt were obtained from Cell Signaling Technology (Beverly, MA, USA). Stock solutions of Pts and TMS, at 100 mM in DMSO, were stored at −80 °C before use.

Cell Culture

Primary HUVECs were purchased from Cascade Biologics Inc. (Portland, OR, USA) and used between the passage 3 and 8. HUVECs were grown in endothelial growth supplement-2 medium (Cambrex, Walkersville, MD, USA) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT, USA), streptomycin (100 U/mL) and penicillin (100 U/mL) under an atmosphere of 5 % CO_2 and 95 % humidified air at 37 °C.

Cell Viability Assay

Cell viability was determined by MTT assay. Cells were treated with MTT at 0.5 mg/mL. The purple formazan crystals were dissolved in DMSO, and determined on an automated microplate spectrophotometer (Molecular Devices, Silicon Valley, CA, USA) at 570 nm.

Serum Starvation

For Western blot analysis and NO assay, confluent HUVECs grown in 6-well and 12-well plates were serum-starved in phenol red-free HBSS supplemented with *L*-arginine (0.1 mM) for 30 min, followed by stimulation with various concentrations of agents. This serum starvation was to prevent the deactivation of the normal enzyme activity by serum proteins, a procedure that is commonly used in the enzyme activity studies [15].

Western Blot Analysis

Equal amounts of cytosolic extracts were electroblotted onto a nitrocellulose membrane, following separation using 8~12 % sodium dodecylsulfate-polyacrylamide gel electrophoresis. The blot was probed using the primary antibodies, and HRP-conjugated anti-IgG antibodies were used as the secondary antibodies to detect antibody-specific protein bands by WESTSAVE-Up® (AbFrontier, Seoul, Korea), a chemiluminescence detection kit. The band density that represents the phosphorylation and expression of eNOS, Akt and ERα

protein were determined by Image J (image processing) software program (NIH, Bethesda, MD, USA) by a single investigator.

NO Assay

NO production was determined by measuring the sum concentration of nitrite and nitrate, the final products of NO, in culture supernatants using a fluorimetric assay kit (Cayman Chemical) following the manufacturer's protocol.

Statistical Analysis

Results of all experiments are expressed as the mean±standard error (SE) of multiple experiments ($n \geq 3$). Statistical analyses were performed using Student's two-tailed *t*-test or one-way ANOVA followed by Dunnett's multiple comparison with GraphPad Prism™ (GraphPad, San Diego, CA, USA). A value of $P<0.05$ was considered statistically significant.

Results and Discussion

Pts, but not TMS, Stimulates Endothelial NO Production via eNOS Phosphorylation

We treated HUVECs with different concentrations of Pts or TMS (their chemical structures shown in Fig. 1a). Pts did not significantly influence the viability of HUVECs in the dose range between 1 and 20 μM (Fig. 1b). However, TMS at the concentration higher than 10 μM proved to be cytotoxic at 24 h after TMS administration (Fig. 1b). In fact, TMS is reported to exert anti-microtubule activity [16], thereby resulting in cell growth inhibition and apoptosis. Thus, through the entire experiments, the maximum concentration of Pts or TMS was limited to 10 μM. At the non-cytotoxic concentrations, Pts increased nitrite/nitrate production in a concentration-dependent manner (Fig. 2a). To verify that the increase in nitrate/nitrite production in response to Pts specifically reflected NO release from eNOS, we examined the effects of the NOS inhibitor L-NAME. Nitrite/nitrate production by Pts was abrogated by pretreatment of HUVECs with L-NAME (Fig. 2a), suggesting that nitrite/nitrate production by Pts reflected NO release from eNOS. To determine whether nitrite/nitrate production was attributable to eNOS activation, we tested the effects of Pts on eNOS phosphorylation on residue Ser1177 by Western blot analysis. As shown in Fig. 2b, treatment of HUVECs with Pts resulted in a concentration-dependent increase in eNOS phosphorylation. Prolonged treatment of HUVECs with Pts for up to 24 h slightly enhanced NO production (EMS Fig. 1a), which is probably due in part to rapid metabolic inactivation of Pts. Next, we examined whether TMS could also stimulate NO production

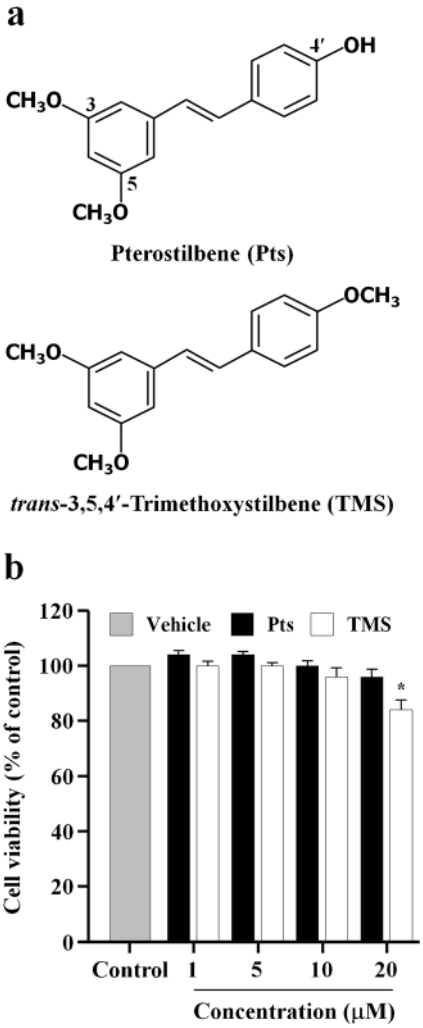

Fig. 1 Chemical structures of Pts and TMS (**a**). Effects of Pts and TMS on cell viability in HUVECs (**b**). Cells were exposed for 24 h to indicated concentrations of Pts or TMS. Data are expressed as mean±SE from three separate experiments. *$P<0.05$ versus vehicle-treated control

in HUVECs. Unlike Pts, TMS had no significant effect on nitrite/nitrate production and eNOS phosphorylation (Fig. 2c and d). Also, prolonged treatment with TMS had no effect on NO production (EMS Fig. 1a). In structural comparison, Pts has two methoxyl groups and one hydroxyl group, while TMS possesses three methoxyl groups. This structural difference strongly suggests that the 4'-hydroxyl group of Pts is likely to be one of important structural features necessary to stimulate eNOS phosphorylation, at least in part, in HUVECs.

Pts Induces eNOS Phosphorylation Through Activation of PI3K/Akt, but not of ERα

It has been previously reported that resveratrol can stimulate eNOS phosphorylation through ERα activation in endothelial cells [14]. Thus, we examined whether Pts, similarly to resveratrol, could also activate ERα in HUVECs. Surprisingly,

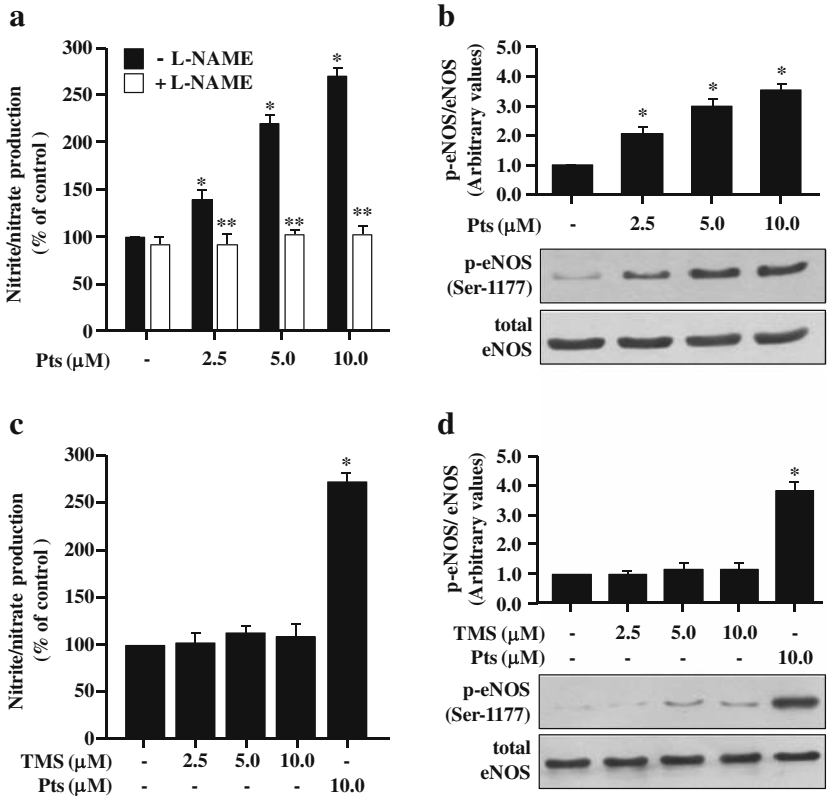

Fig. 2 Effects of Pts and TMS on NO production (**a, c**) and eNOS phosphorylation (**b, d**) in HUVECs. Cells were pre-incubated for 1 h with or without L-NAME (100 μM), and then exposed for 1 h (**a, c**) or 30 min (**b, d**) to indicated concentrations of Pts or TMS or to 10 μM Pts. Relative changes in eNOS phosphorylation were assessed by scanning densitometry (*upper panel*). Representative blots are shown (*lower panel*). The experiment was repeated three times, and data are expressed as mean±SE. **P*<0.05 versus vesicle-treated control and ***P*<0.05 versus L-NAME-untreated group

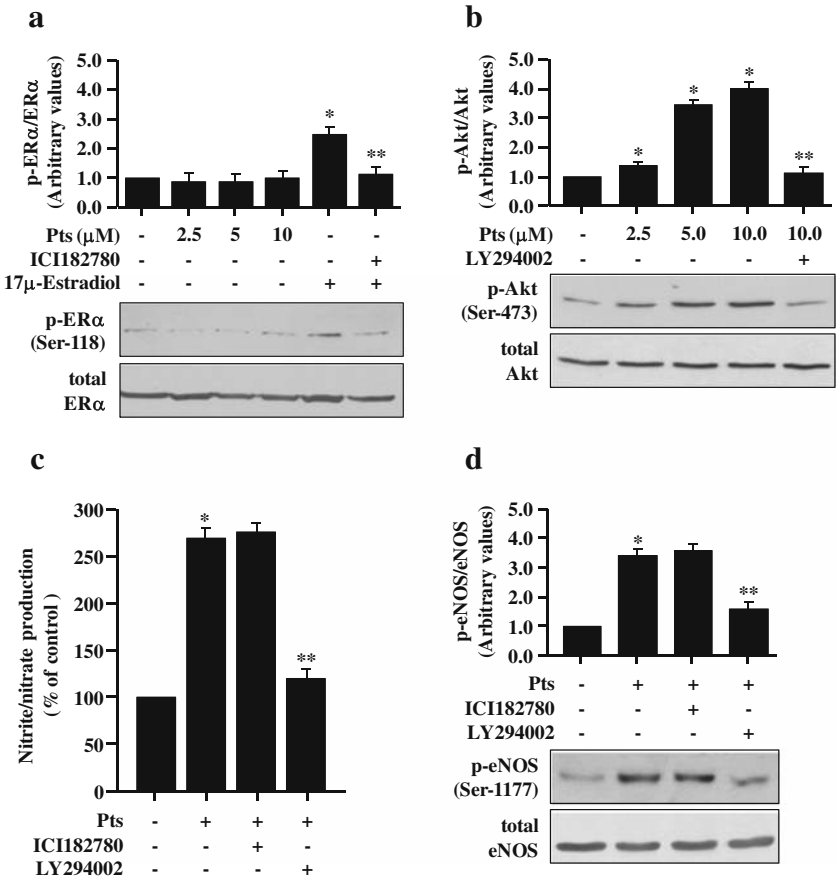

Fig. 3 Effects of Pts on ERα (**a**) and Akt (**b**) phosphorylation and effects of ICI182780 and LY294002 on Pts-induced NO production (**c**) and eNOS phosphorylation (**d**) in HUVECs. Cells were pre-incubated for 1 h with or without ICI182780 (100 nM) or LY294002 (10 μM), and then exposed for 30 min (**a, b**) or 1 h (**c, d**) to indicated concentrations of Pts or 17β-estradiol (100 nM) or to 10 μM Pts. Relative changes in ERα and Akt phosphorylation were assessed by scanning densitometry (*upper panel*). Representative blots are shown (*lower panel*). The experiment was repeated three times, and data are expressed as mean±SE. **P*<0.05 versus vesicle-treated control and ***P*<0.05 versus Pts-treated group

Pts had no significant effect on ERα phosphorylation; the positive control 17-β-estradiol (ERα agonist), however, significantly induced ERα phosphorylation on residue Ser118, which was significantly abolished by the ERα antagonist ICI182780 (Fig. 3a). Thus, eNOS phosphorylation in response to Pts is unlikely to be mediated by ERα activation, at least in part, under our experimental conditions.

It has been also reported that some polyphenolic compounds can activate eNOS through PI3K/Akt pathway [17–21]. To elucidate one of possible upstream signaling pathways involved in Pts-mediated eNOS phosphorylation and subsequent NO release, we examined the activation of PI3K/Akt in Pts-treated HUVECs. Western blot analysis using an antibody specific to phosphor-Ser473 of Akt showed that Pts treatment caused a concentration-dependent phosphorylation of Akt, which was significantly abolished by the PI3K/Akt inhibitor LY294002 (Fig. 3b). We also examined the effect of Pts on NO production and eNOS phosphorylation in the presence of an ERα antagonist and a PI3K/Akt inhibitor. As expected, nitrite/nitrate production and eNOS phosphorylation in response to Pts were significantly attenuated in the presence of LY294002 (Fig. 3c), but not in the presence of ICI182780 (Fig. 3d). Based on these data, eNOS phosphorylation induced by Pts is likely to be mediated by activation of the PI3K/Akt signaling pathway. A similar mechanism of eNOS phosphorylation has been also observed in response to the polyphenolic compounds, including epigallocatechin-3-gallate [17] and luteolin [18], and the polyphenol-rich products, including a grape seed extract [19], grape skin extract [20], and strawberry powder [21]. It is noteworthy that the effect of Pts on stimulating NO production was comparable to that of luteolin that was served as a positive control (EMS Fig. 1b). However, the ant-microtubule combretastatin A4 that has structural similarity to TMS had no effect on NO production (EMS Fig. 1b). It is also noteworthy that repeated and long-term treatment of HUVECs with physiological concentrations of resveratrol results in increased eNOS expression [22, 23], raising the possibility that repeated and long-term treatments with Pts may up-regulate eNOS expression.

Conclusions

This study, for the first time, demonstrates that Pts, an active constituent of blueberries, is capable of inducing a concentration-dependent NO release via eNOS phosphorylation in endothelial cells. This effect is mainly mediated by activation of the PI3K/Akt signaling pathway, although the activation of other kinases cannot be ruled out. The potential efficacy of Pts could aid in the prevention of vascular diseases characterized by endothelial dysfunction. However, it is important to note that the effects of Pts described in this study were obtained with high concentrations that could not be measured in human plasma after dietary consumption, such as intake of blueberries containing Pts. It is suggested that a pharmacological intervention for Pts treatment could be necessary to reach higher plasma levels. It is noteworthy that a recent study has demonstrated that Pts reduces blood pressure in human subjects at 250 mg/day doses [24]. Considering the effect of Pts on stimulating endothelial NO production that contributes to the regulation of blood pressure, such an antihypertensive effect of Pts may be associated with increased endothelial NO release.

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Conflict of interest The authors declare no conflicting interests or financial disclosures.

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