Piceatannol-3'-O-β-D-glucopyranoside as an active component of rhubarb activates endothelial nitric oxide synthase through inhibition of arginase activity

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Abbreviations: ArgI, arginase I; ArgII, arginase II; DAF-FM, 4-amino-5-methylamino-2',7'-difluorescein; DHE, dihydroethidine; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; IC_{50}, half maximal inhibitory concentration; L-NAME, nitro-L-arginine methyl ester; NO, nitric oxide; NOx, nitrite and nitrate; PG, piceatannol-3'-O-β-D-glucopyranoside

Abstract

Arginase competitively inhibits nitric oxide synthase (NOS) via use of the common substrate L-arginine. Arginase II has recently reported as a novel therapeutic target for the treatment of cardiovascular diseases such as atherosclerosis. Here, we demonstrate that piceatannol-3'-O-β-D-glucopyranoside (PG), a potent component of stilbenes, inhibits the activity of arginase I and II prepared from mouse liver and kidney lysates, respectively, in a dose-dependent manner. In human umbilical vein endothelial cells, incubation of PG markedly blocked arginase activity and increased NOx production, as measured by Griess assay. The PG effect was associated with increase of eNOS dimer ratio, although the protein levels of arginase II or eNOS were not changed. Furthermore, isolated mice aortic rings treated with PG showed inhibited arginase activity that resulted in increased nitric oxide (NO) production upto 78%, as measured using 4-amino-5-methylamino-2',7'-difluorescein (DAF-FM) and a decreased superoxide anions up to 63%, as measured using dihydroethidine (DHE) in the intact endothelium. PG showed IC_{50} value of 11.22 μM and 11.06 μM against arginase I and II, respectively. PG as an arginase inhibitor, therefore, represents a novel molecule for the therapy of cardiovascular diseases derived from endothelial dysfunction and may be used for the design of pharmaceutical compounds.

Keywords: arginase; endothelium, vascular; nitric oxide synthase type III; superoxides; 3,3',4,5'-tetrahydroxystilbene

Introduction

The endothelium plays a central role in overall vascular homeostasis by regulating vasoreactivity, platelet activation, leukocyte adhesion, and smooth muscle cell proliferation and migration. Endothelial nitric oxide (NO), an important vasoprotective molecule, is a major modulator of these effects, and impaired NO signaling associated with endothelial function is considered an early marker of the atherogenic process. Arginase competitively inhibits nitric oxide synthase (NOS) via use of the common substrate L-arginine (Morris *et al.,* 1998; Berkowitz *et al.,* 2003; Simon *et al.,* 2003; Holowatz *et al.,* 2006; Steppan *et al.,* 2006; Peyton *et al.,* 2009). Arginase is present in 2 isoforms: arginase I, the hepatic isoform; and arginase II, the extrahepatic isoform; each of which is encoded by a distinct gene. The expression and function of arginase I in macrophages, hepatocytes, and vascular smooth muscle cells, is stimulated by lipopolysaccharide (LPS), IL-13, altered oxygen tension, and balloon dilatation of coronary arteries (Modolell *et al.,* 1995; Louis *et al.,* 1998; Que *et al* 1998; Klasen *et al.,* 2001; Chicoine *et al.,* 2004; Morris *et al.,* 2004; Ryoo *et al.,* 2006; Nelin *et al.,* 2007). The activation and expression of endothelial arginase II can also be induced by a variety of vascular insults, including OxLDL, LPS, TNF-α, IFN-β, 8-bromo-cGMP, and hypoxia (Morris *et al.,* 1998; Que *et al.,* 1998; Chicoine *et al.,* 2004; Ryoo *et al.,* 2006; Nelin *et al.,* 2007). Arginase inhibition actively augments NO production and reportedly has beneficial effects on normal cardiac function and on vascular dysfunction typical of atherogenesis,

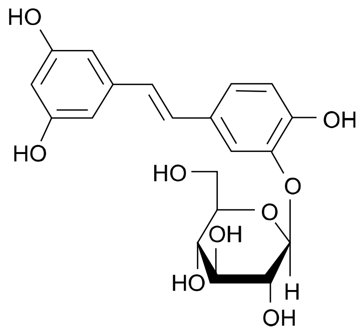

Figure 1. Structure of piceatannol-3'-O-β-D-glucopyranoside (PG).

aging, and erectile dysfunction, and sickle cell disease (Bivalacqua *et al.,* 2001; Berkowitz *et al.,* 2003; Morris *et al.,* 2004; Steppan *et al.,* 2006; White *et al.,* 2006; Bivalacqua *et al.,* 2007; Hsu *et al.,* 2007; Xu *et al.,* 2007).

Rhubarb is an important medicinal original plant that has been used in traditional medicine as a remedy for the blood stagnation and a purgative agent. Rhubarb is the rhizome of *Rheum undulatum* L. commonly distributed in Asia and many components of rhizome has been demonstrated as anthraquinone derivatives or stilbenes such as emodin, chrysophanol, physcion, resveratrol, rhapontigenin, rhaponticin, and piceatannol (Xu *et al.,* 2007; Ngoc *et al.,* 2008). These compounds reportedly possess diverse biological activities such as anti-allergic (Matsuda *et al.,* 2004), anti-diabetic (Choi *et al.,* 2005), antioxidant (Matsuda *et al.,* 2001), and vasorelaxant activities (Moon *et al.,* 2006). Piceatannol is an important component in rhubarb extract and was recently found to possess various activities such as inhibition of C-jun N-terminal kinase activation (Jang *et al.,* 2009), anti-carcinogenic activity (Vo *et al.,* 2009), and inhibition of PDGF-BB-induced VSMC proliferation and migration (Choi *et al.,* 2009). However, the biological activity and the molecular target for piceatannol-3'-O-β-D-glucopyranoside (PG) have not been investigated.

Thus, we tested whether piceatannol-3'-O-β-D-glucopyranoside(PG) has an inhibitory effect on arginase activity and whether this effect is asso-

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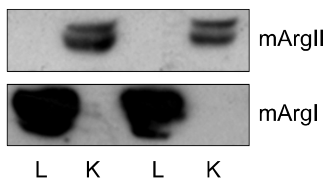

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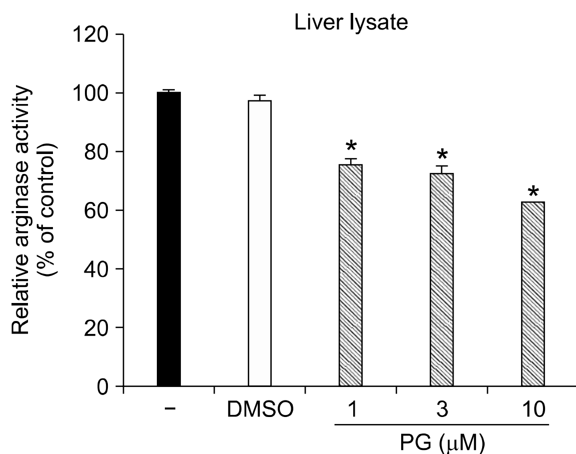

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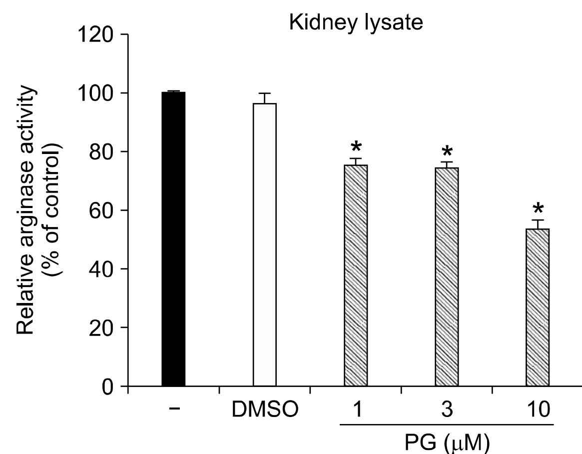


Figure 2. PG decreases the activities of arginase I and II. Arginase solutions prepared from liver (L) and kidney (K) were confirmed to express arginase I or II by Western blot analysis (A). Arginase I was predominantly expressed in liver while arginase II was in kidney. Incubation of different concentrations PG significantly decreased arginase I (B, liver lysate) and II (C, kidney lysate) activities. DMSO (10 μM) was used as a control. * vs. untreated, $P < 0.01$, $n = 12$.

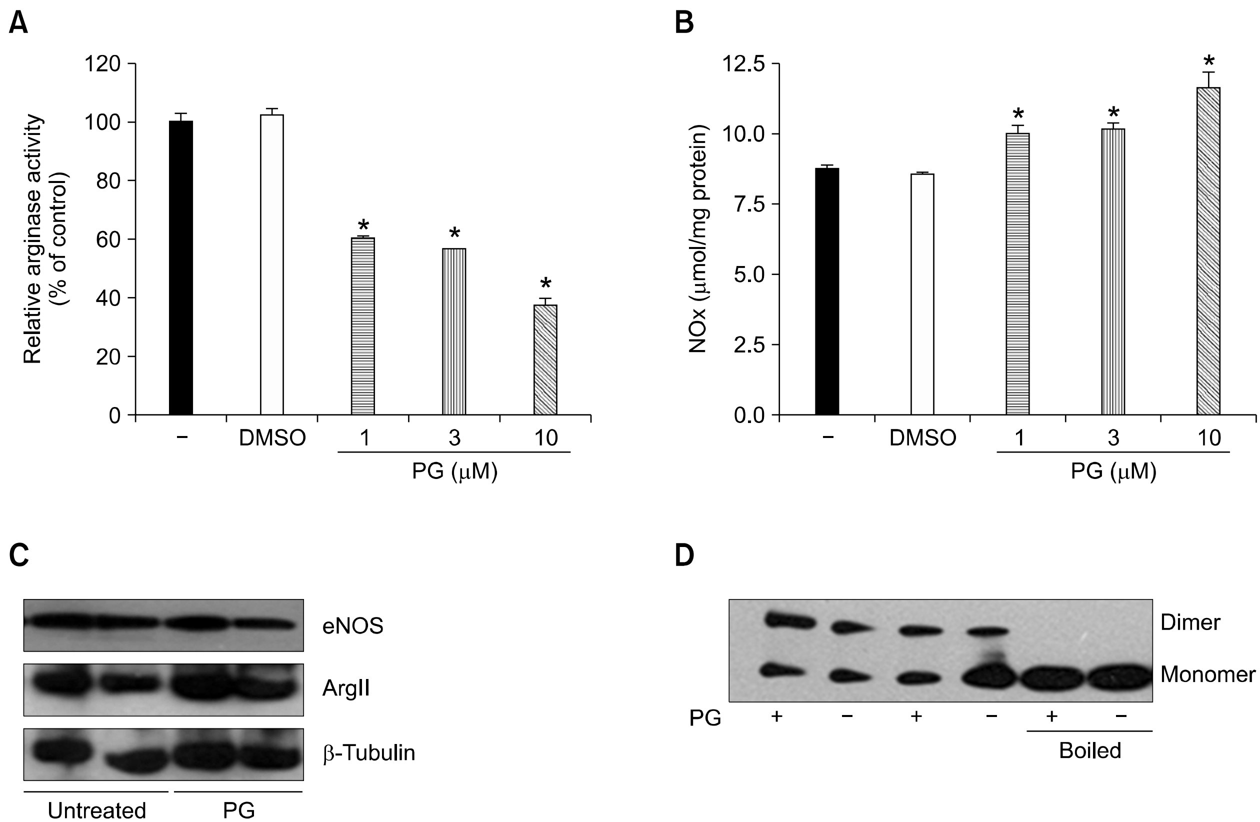


Figure 3. Inhibition of arginase activity is associated with a reciprocal increase in endothelial NOx production in HUVECs. HUVECs were incubated with different concentrations of PG for 12 h. PG inhibited arginase activity in HUVECs in a dose-dependent manner (A, * vs. untreated, $P < 0.01$, $n = 4$) and reciprocally increased NOx production (B, * vs. untreated, $P < 0.01$, $n = 4$). Protein levels of arginase II and eNOS were analyzed after overnight incubation with PG (10 μM). Arginase II and eNOS protein levels were not significantly changed by PG treatment (C, $n = 4$). PG incubation (10 μM, 6 h), however, induced eNOS dimerization in low-temperature SDS-PAGE and western blot analysis. Boiled samples were used as a control.

ciated with reciprocally regulation of NOx and ROS production in the endothelium.

Results

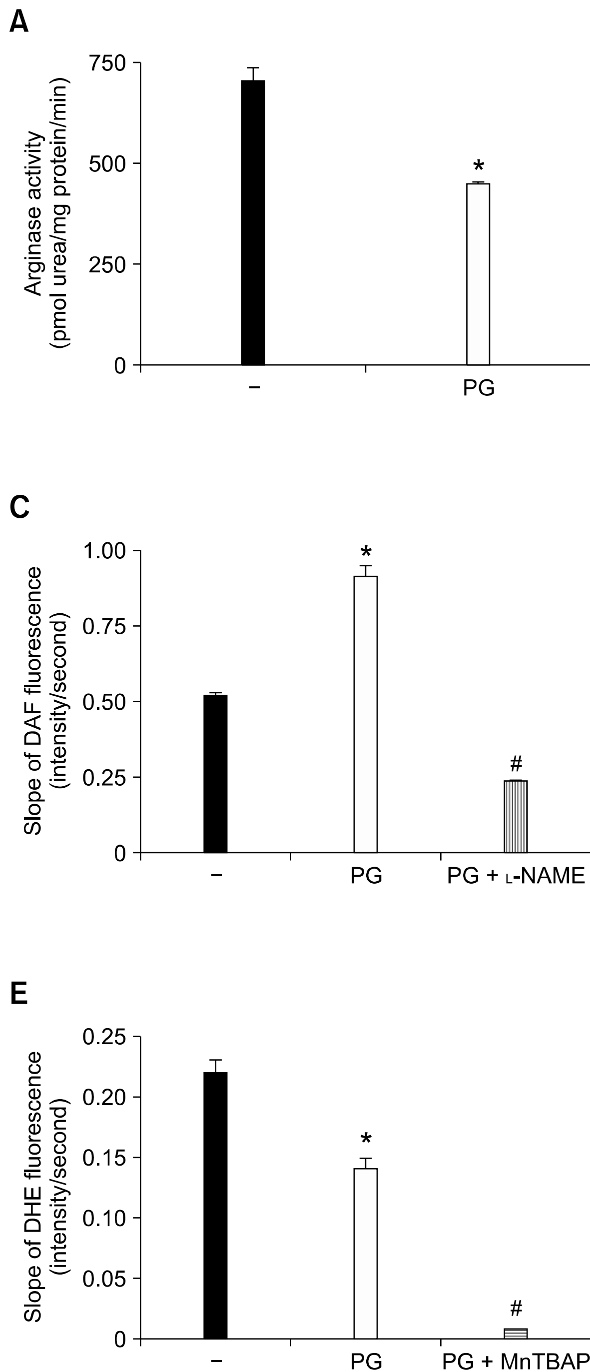
Arginase activity was decreased with treatment of piceatannol-3'-O-β-D-glucopyranoside (PG) treatment

We first prepared arginase enzyme solutions for arginase I and arginase II from the lysates of mouse livers and kidneys, respectively, and the presence of specific arginase isoforms was detected by Western blot analysis. As shown in Figure 1A, arginase I was predominantly expressed in liver lysates while arginase II was in the kidney. In liver lysates, incubation of different concentrations of PG significantly decreased arginase I activity (Figure 2B, 75 ± 5% at 1 μM, 72 ± 7% at 3 μM, 62 ± 1% at 10 μM) compared to untreated control (100±9%). In kidney lysates, the residual arginase activities after incubation of 1, 3 and 10 μM PG were 75 ± 6, 74 ± 5, and 53 ± 8%, respectively (Figure 2C). These data indicated that PG has a strong and specific inhibitory effect on both arginase I and II even if PG has no selectivity for a specific arginase isoform.

PG treatment reciprocally increased NOx production

Given recent data suggesting that arginase reciprocally regulates NOS activity by limiting bioavailability of L-arginine (Ryoo *et al* 2006), we tested whether the PG-mediated decrease in arginase activity is associated with increased NOx production in HUVECs. As demonstrated in Figure 3, incubation of HUVECs with PG for 12 h markedly decreased arginase activity (A, PG vs. untreated, 60 ± 3% at 1 μM, 56 ± 1% at 3 μM, 37 ± 9% at 10 μM vs. 100 ± 9%, $P < 0.01$) and increased NOx production (B, PG vs. untreated, 9.99 ± 0.42% at 1 μM, 10.08 ± 0.43% at 3 μM, 11.55 ± 1.07 at 10 μM vs. 8.73 ± 0.3 μmol/mg protein, $P < 0.01$).

To further test the effect of PG on the protein



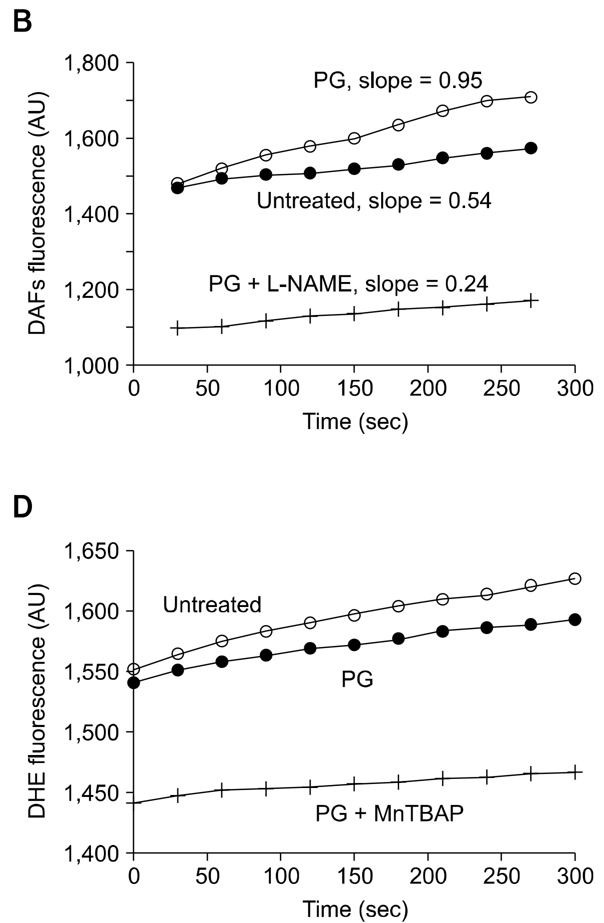


Figure 4. Arginase inhibition by PG increases NO production and decreases ROS generation in mouse aorta. Incubation of mice aortic rings with PG (40 μM, overnight≒16 h) resulted in significant decrease in arginase activity (A, * vs. untreated, $P < 0.01$, $n = 4$). (B) The pretreated aorta were loaded with DAF (5 μM) followed by measurement of fluorescence (endothelial side up). The graph shows representative traces of DAF fluorescence in PG- or PG plus L-NAME (10 μM)-treated aorta. (C) The slope of DAF fluorescence was monitored and then determined (* vs. untreated, $P < 0.01$; # vs. PG, $P < 0.01$; $n = 4$). (D) ROS production from the aorta was traced after preloading with DHE (5 μM). MnTBAP (10 μM) was used as a superoxide scavenger. (E) The slope of DHE fluorescence was determined based on cumulative data (* vs. untreated, $P < 0.01$; # vs. PG, $P < 0.01$; $n = 4$ mice).

levels of arginase II and eNOS, Western blot analysis was performed with PG-treated HUVECs. As demonstrated in Figure 3C, PG had no significant effect on the protein levels of arginase II or eNOS. Next, we tested eNOS dimerization to elucidate a mechanism associated with increased NOx production by PG treatment. Interestingly, PG treatment (6 h) resulted in increased ratio of eNOS dimer/monomer from 0.70 ± 0.15 to 1.02 ± 0.09 (Figure 3D). Therefore, these data indicate that increased NOx production upon PG treatment was dependent on the increased bioavailability of L-arginine resulting from the inhibition of arginase activity, which itself is associated with eNOS dimerization (Ryoo *et al.,* 2008).

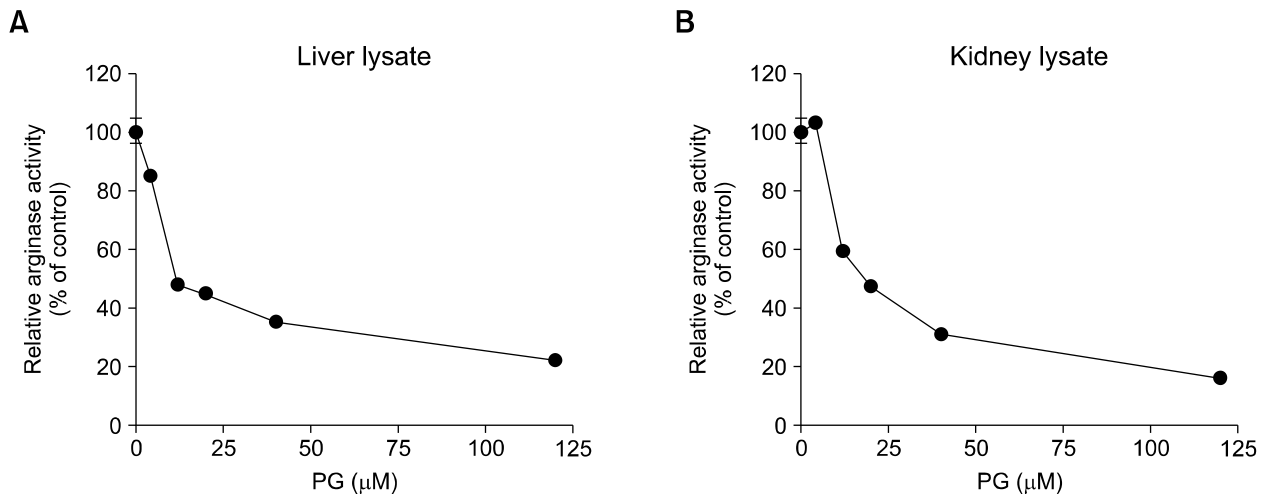


Figure 5. PG inhibits the activities of arginase I and II in a dose-dependent manner. Arginase I (A) and II (B) activities were measured with increasing doses of PG. IC_{50} values of PG were 11.22 μM and 11.06 μM against arginase I and II, respectively. Relative enzyme activity is an average from 3 different experiments.

PG inhibits arginase activity, increases NO production, and decreases ROS production in mice aorta

We next wished to determine whether or not increased NOx production in HUVECs translates into redox regulation in the endothelium of aortic tissue. Therefore, we measured the intensity of DAF-AM and DHE fluorescence at different time intervals. At first, PG treatment for overnight decreased arginase activity in aorta isolated from anethesized mice (Figure 4A, untreated vs. PG, 704 ± 49 vs. 445 ± 14 pmol Urea/mg protein/min, $P < 0.01$). We next tested whether PG-dependent arginase inhibition increased in NO production using an NO-sensitive fluorescence dye, DAF-FM diacetate (see materials and methods). Incubation of aorta with PG markedly increased the average slope of DAF fluorescence (Figure 4C; untreated vs. PG, 0.51 ± 0.02 vs. 0.91 ± 0.07). On the other hand, incubation of N^G-Nitro-L-arginine methyl ester (L-NAME) acutely decreased the slope of DAF fluorescence (slope = 0.23 ± 0.01). This is consistent with previous observations in HUVECs. The representative traces of DAF fluorescence in the aortic endothelium were shown in Figure 4B.

To determine whether increased NO production upon arginase inhibition contributes to ROS reduction, we measured $O2^{\cdot -}$ generation using the $O2^{\cdot -}$-sensitive dye DHE in the endothelia of PG-treated aorta. The time-dependent intensity of DHE fluorescence was decreased by incubation of PG compared to untreated control (Figure 4E. slope of DHE fluorescence; * vs. untreated, 0.14 ± 0.01 vs. 0.22 ± 0.01). The DHE signal was completely quenched with MnTBAP (slope < 0.01). The representative traces of DHE fluorescence are shown in Figure 4D.

PG inhibits arginase activity in a dose-dependent manner

Given that PG inhibited the arginase activity of both isoforms, we wished to determine the efficacy of PG on arginase isoforms. Arginase activity was measured in the presence of different concentration of PG (from 0 to 120 μM) using liver lysate and kidney lysate. The 50% inhibitory concentrations (IC_{50}) were 11.22 μM for the liver lysate and 11.06 μM for kidney lysate. The values were obtained using the software of Graphpad prizm 4.0 (Figure 5).

Discussion

With the idea that arginase modulates NOx production by NOS through limiting L-arginine substrate is emerging as a general mechanism for NOS regulation and appear to contribute to the pathobiology of a number of disease processes in which NO is dysregulated, we here demonstrate for the first time that PG, as an active component of Rhubarb, inhibits arginase activity and reciprocally increases NOx production.

PG-dependent inhibition of arginase activity contributed to increase of NO production in both HUVECs and endothelium of isolated mice aorta (Figures 3 and 4). These results are consistent

with previous observations that arginase inhibition accentuates NO release in rat aortic endothelium (Berkowitz *et al.,* 2003), bovine pulmonary endothelial cells (Chicoine *et al.,* 2004), and a porcine coronary artery model (Zhang *et al.,* 2001). The PG-mediated increase in NO production may result in eNOS coupling through increased L-arginine availability. During eNOS uncoupling, electrons flowing from the reductase domain in the heme to molecular oxygen rather than L-arginine, resulting in production of $O2^{\cdot -}$ instead of NO. There are number of circumstances in which this may occur specifically tetrahydrobiopterin cofactor deficiency and relative L-arginine deficiency. As shown in Figures 3 and 4, the availability of substrate, cofactors as well as local eNOS microdomain concentration of L-arginine, rather than the expression level and abundance of the eNOS enzyme were critical to NO production. Regarding the physiological role of arginase in reciprocal NO regulation, arginase isoforms play important roles in regulating the synthesis of polyamines and proline (Li *et al.,* 2001, 2002) and arginase inhibition blocked HUVECs proliferation, which is an emerging phenomenon associated with angiogenesis (Faffe *et al.,* 2005). Furthermore, reciprocal regulation of NOS by arginase has been demonstrated in cells and organs in which NO is an important signaling molecule including the endothelium, cardiac myocytes, penis, airway, skin, and inflammatory cells(Bivalacqua *et al.,* 2001; Berkowitz *et al.,* 2003; Morris *et al.,* 2004; Steppan *et al.,* 2006; White *et al.,* 2006; Bivalacqua *et al.,* 2007; Hsu *et al.,* 2007; Xu *et al.,* 2007; Kim *et al.,* 2008). It was demonstrated that arginase II activity is upregulated in atherosclerosis-prone mice and is associated with impaired endothelial NO production, endothelial dysfunction, vascular stiffness, and ultimately, aortic plaque development. Conversely, inhibition of endothelial arginase or deletion of the arginase II gene enhances NO production, restores endothelial function and aortic compliance, and reduces plaque burden. Therefore, arginase II representes a novel target for the prevention and treatment of atherosclerotic vascular disease (Ryoo *et al*., 2008). Furthermore, upregulation of arginase activity contributes to endothelial dysfunction in systematic and pulmonary hypertension, aging, diabetes and erectile dysfunction and to bronchodilatory dysfunction in asthma.

With together arginase inhibitory activity as presented in this report, Ngoc *et al.* (2008) also showed other biological activities of PG. PG inhibited lipoxygenase activity upto 66% at the concentration of 100 μM and IC_{50} value was 69 μM, although resveratrol had much potent activity, such that 92% inhibition at the same concentration and 12 μM at IC_{50} value. Furthermore, they showed that PG had a potency as a radical scavenger in $ABTS^{\cdot -}$ radical scavenging assay in which resveratrol exhibited the most potent scavenging activity.

In summary, we present a novel molecule, piceatannol-3'-O-β-D-glucopyranoside (PG), that inhibited arginase activity and increased NO production in HUVECs. PG showed an IC_{50} of about 11 μM against arginase I and II. Furthermore, PG increased NO production and decreased ROS production in isolated mice aorta. Although the inhibitory potency of PG against arginase activity was a little higher compared to boronic acid analogues, identification of a new moiety that inhibits arginase activity would be very useful for the development of a new pharmaceutical compound. The continued development of the derivatives with increased specificity and selectivity against arginase isoforms may lead to novel therapies for the treatment of various diseases from NO dysregulation.

Methods

Materials

Arginase lysates were prepared from livers and kidneys of anesthetized C57BL/6 mice. MnTBAP (Mn(III) Tetra (4-benzoic acid) porphyrin chloride) and L-NAME (N^G-nitro-L-arginine methyl ester) were obtained from Calbiochem. All reagents were purchased from Sigma unless otherwise stated.

PG preparation

PG was prepared as previously described (Ngoc *et al*., 2008). Briefly, the dried and milled rhizomes of rhubarb were extracted with ethanol. The extract was dried and then resolved again in ethylacetate. The ethylacetate-soluble fraction was diluted with acetone and subjected to silica gel column chromatography. The active fraction eluted with the mixture of chloroform and methanol was further purified with ODS column, eluting with methanol and water (1:2 to 3:1) to afford PG.

Cell culture

HUVECs were purchased from Cascade Biologics and maintained as supplier's protocol in Medium230 plus low-serum growth supplement (LSGS) at 37°C in 5% CO_2.

Arginase activity assay

Tissue lysates were prepared using lysis buffer (50 mM Tris-HCl, pH7.5, 0.1 mM EDTA and protease inhibitors) by homogenization at 4°C followed by centrifugation for 20

min at 14,000 × *g* at 4°C. The supernatants were used to assay for arginase activity as previously described (Ryoo *et al*., 2006).

NOx measurement

NO was estimated by Griess reaction based upon the concentration of nitrate/nitrite (NOx) after conversion of nitrate to nitrite by nitrate reductase using the Nitric oxide assay kit (Calbiochem). The concentration of NOx in HUVECs was expressed as μmol/mg protein.

Western blot analysis

The livers or kidneys from C57BL/6 mice (10 weeks) were homogenized in the buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 μg/ml of aprotinin, 1 mM phenylmethylsulfonylflouride, 1 mM sodium orthovanadate, and 1 mM NaF) and centrifuged for 30 min at 14,000 × *g*. The protein amount of the supernatant was analyzed by the Bradford method. Protein (100 μg) were separated in a 10% SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). The blots were incubated with a monoclonal anti-arginase I (Santa Cruz), anti-arginase II (Santa Cruz), anti-endothelial nitric oxide synthase(eNOS, BD Bioscience), or anti-β-tubulin (BD bioscience) antibodies followed by the secondary antibody (Amersham). The signals were detected using an enhanced chemiluminescence detection reagent with X-ray films.

Determination of eNOS dimerization

Dimers and monomers of eNOS were separated using low-temperature SDS-PAGE as previously described (Takimoto *et al*., 2005). Band intensities were analyzed using NIH ImageJ Software.

Estimation of NO or ROS generation in isolated mice aorta using DAF-FM or DHE

Mice aortic rings were isolated and incubated overnight at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) medium containing 2% FBS and antibiotics (1×) in the presence of picaetannol-3'-O-β-D-glucopyranoside (50 μmol/L) (White *et al*., 2006). The aorta cut longitudinally and pinned to the bottom of a silgard-coated chamber (endothelial layer on top) filled with HEPES buffer (NaCl 120 mM, KH_2PO4 2.6 mM, KCl 4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 0.6 mM, HEPES 25 mM, glucose 14 mM, pH7.4). The chamber was allowed to equilibrate into the heating stage for 30 min at 37°C. The chamber allowed for static bath conditions during fluorescence measurements. Tissue background along with DAF-FM (4-Amino-5-methylamino-2',7'-difluorofluorescein) diacetate or DHE (dihydroethidine) fluorescence were measured using an Olympus 10× objective with optimized excitation and emission wavelength (DAF-FM, 470/525 nm; or DHE, 470/580), an intensified camera (Luca 658M-TL), and a custom image acquisition program (Cell software, Olympus). Following initial equilibrium, background fluorescence was recorded and aorta was allowed to return to room temperature for 15 min. The aorta were then loaded with 5 μM DAF-FM or 5 μM DHE (molecular Probes) in HEPES buffer for 45 min followed by washout of DAF-FM or DHE and a 20 min equilibrium period at 37°C. Fluorescence intensity was averaged (5 frames, 2 × 2 binning) from the entire field of view and recorded by the acquisition program. Changes in DAF-FM or DHE fluorescence were recorded once after washout of DAF-FM or DHE in order to establish baseline changes in intensity and then again following treatment with L-NAME (10^{-5} mol/L) or MnTBAP (10^{-5} mol/L). The slopes of changes in fluorescence over time were determined by linear regression in Origin (version 7.5, OrignLab Corp, Northampton, MA) and used for statistical comparison.

Statistics

All data are represented as mean ± S.D. of at least four independent experiments. An unpaired Student's *t*-test or 1-way ANOVA was used to assess significant differences. A value of $P < 0.05$ was accepted as significant.

Acknowledgements

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