A Novel arginase inhibitor derived from *Scutellavia indica* restored endothelial function in ApoE-null mice fed a high cholesterol diet

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Abbreviations: NO, nitric oxide; eNOS, endothelial nitric oxide synthase; TDF, (2S)-5,2’,5’-trihydroxy-7,8-dimethoxy flavanone; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; Ach, acetylcholine; HCD, high cholesterol diet; ND, Normal diet; SNP, sodium nitroprusside; WT, wild type; PE, phenylephrine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; DHE, dihydroethidine; DAF, 4-amino-5-methylamino-2’,7’-dihydroethidium; iNOS, inducible nitric oxide synthase; L-NAME, L-N6-Nitroarginine methyl ester; ApoE-/-, ApoE-null; HSP90, heat shock protein 90; Emax, maximal response; EC50, half maximal effective concentration.
Abstract

Elevated endothelial arginase activity decreases nitric oxide (NO) production by competing with the substrate L-arginine in previous reports and reciprocally regulating endothelial nitric oxide synthase (eNOS) activity. Thus, arginase inhibitors may help treat vascular diseases associated with endothelial dysfunction. A screening of metabolites from medicinal plants revealed that (2S)-5,2',5'-trihydroxy-7,8-dimethoxy flavanone (TDF) was a non-competitive inhibitor of arginase. We investigated whether TDF reciprocally regulated endothelial NO production and its possible mechanism. TDF non-competitively inhibited arginase I and II activity in a dose-dependent manner. TDF incubation decreased arginase activity and increased NO production in human umbilical vein endothelial cells (HUVECs) and isolated mouse aortic vessels and reduced reactive oxygen species (ROS) generation in the endothelium of the latter. These TDF-mediated effects were associated with increased eNOS phosphorylation and dimerization but not changes in protein content. Endothelium-dependent vasorelaxant responses to acetylcholine (Ach) were significantly increased in TDF-incubated aortic rings and attenuated by incubation with soluble guanylyl cyclase inhibitor. Phenylephrine-induced vasoconstrictor responses were markedly attenuated in TDF-treated vessels from wild type mice. In atherogenic-prone ApoE−/− mice, TDF attenuated the high cholesterol diet (HCD)-induced increase in arginase activity, which was accompanied by restoration of NO production and reduction of ROS generation. TDF incubation induced eNOS dimerization and phosphorylation at Ser1177. In addition, TDF improved Ach-dependent vasorelaxation responses and attenuated U46619-dependent contractile responses but did not change sodium nitroprusside (SNP)-induced vasorelaxation or N-NAME-induced vasoconstriction. The findings suggested that TDF may help treat cardiovascular diseases by reducing pathophysiology derived from HCD-mediated
endothelial dysfunction.

Introduction

Vascular disease is a major cause of mortality and morbidity worldwide. Current investigations have shown an association between vascular pathology and decreased bioavailability of nitric oxide (NO). The NO generated by endothelial nitric oxide synthase (eNOS) in the vascular endothelium is a vasoprotectant that regulates vasoreactivity, activation of platelets, expression of adhesion molecules, infiltration of monocytes into the intima, apoptosis of endothelial cells, and proliferation of smooth muscle cells. This regulation ultimately leads to reduced plaque formation and improved maintenance of vascular tone and integrity. Impaired endothelial function causes abnormalities in the arterial wall and is an early and critical event in atherosclerosis (Li and Forstermann, 2009).

The eNOS produces NO by hydrolyzing L-arginine. However, L-arginine can also be converted to L-ornithine and urea by arginase, and increasing arginase activity may induce endothelial dysfunction by decreasing eNOS activity via substrate depletion (Berkowitz et al., 2003). Arginase I and II are distinct enzymes encoded by different genes (Haraguchi et al., 1987; Morris et al., 1997). Arginase I catalyzes the final step of the urea cycle in the liver, although its expression is also induced by hypoxia and lipopolysaccharide (LPS) in a variety of cells and tissues (Chang et al., 1998). Arginase II is the extrahepatic isoform and controls cell proliferation and differentiation in endothelial cells by providing ornithine for polyamine synthesis (Ignarro et al., 2001; Li et al., 2001). Expression of arginase II is induced by factors such as LPS, TNFα, and hypoxia in a variety of tissues (Collado et al., 2006; Louis et al., 1998; Modolell et al., 1995; Morris et al., 1998). Furthermore, the role of arginase II in endothelial dysfunction has recently been observed in animal models of aging (Berkowitz et al., 2003), ischemia-reperfusion injury (Hein et al., 2003; Jung et al., 2010), hypertension
(Johnson et al., 2005; Zhang et al., 2004), balloon vascular injury (Peyton et al., 2009), and atherosclerosis (Ryoo et al., 2006).

Arginase inhibitors with novel structural properties may be useful for the treatment or prevention of vascular diseases associated with endothelial dysfunction. Boronic acid derivatives are currently being investigated as possible therapeutics (Steppan et al., 2013). This study investigated the role of and mechanism behind (2S)-5,2',5'-trihydroxy-7,8-dimethoxy flavanone (TDF), a methanol extract from the plant Scutellaria indica that has been shown to inhibit arginase (Kim et al., 2013), in the restoration of endothelial dysfunction using a rodent model of hyperlipidemia.

**Methods**

**Materials**

The 2(S)-amino-6-boronohexanoic acid (ABH), N^3^-nitro-L-arginine methyl ester (L-NAME), and manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) were purchased from Calbiochem (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

**Preparation of TDF**

*Scutellaria indica* was obtained at Jindo Island, Korea in May 2011, and botanical identification was performed by Prof. Byung Sun Min, an author of this manuscript. The voucher specimen CUD-1523 was deposited at the herbarium of the College of Pharmacy, Catholic University of Daegu, Korea. The flavonoids compounds were isolated as previously described (Kim et al., 2013).

**Cell culture**
Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biologics (Portland, OR) and maintained in Medium 200 containing low serum growth supplement according to the supplier’s protocol.

Animals

Twenty male C57BL/6J wild type (WT) and 20 male ApoE−/− mice (Daehan Biolink Co. Chungbuk, Korea) were obtained at 10 weeks of age and fed a normal diet (ND) and a high cholesterol diet (HCD, D12108C, Research Diet Inc., USA), respectively, for 8 weeks. Aortic rings were incubated with or without TDF at indicated concentrations for 18 hours as previously described (Yoon et al., 2014). The study was approved in accordance with the Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kangwon National University).

Measurement of arginase activity

Arginase activity was assessed by measuring urea content using α-isonitrosopropiophenone as described previously (Ryoo et al., 2006). Briefly, supernatants of extracted cell lysates were prepared by incubation with lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and protease inhibitors) for 30 min at 4°C and centrifugation for 20 min at 14,000 × g at 4°C. Assays on aortic vessel samples were performed following homogenization in lysis buffer.

NO measurement

NO was estimated by the nitrite/nitrate ratio (NOx), calculated using the Griess test to assess the conversion of nitrate to nitrite by nitrate reductase, using a Nitric Oxide Assay kit (Calbiochem.) as previously described (Yoon et al., 2014).

Lucigenin chemiluminescence
Lucigenin-enhanced chemiluminescence (5 μM, lucigenin) was recorded using a luminometer (Monolight™ 3010) (Ryoo et al., 2008).

**Measurement of NO and reactive oxygen species (ROS)**

All experimental procedures using mice were approved by the Institutional Review Board at Kangwon National University. Aortic rings from 10-week-old male C57BL/6 WT mice were prepared for biochemical assays of arginase activity or fluorescent probe labeling of superoxide (dihydroethidine [DHE], 1 μmol/L, 5 min) or NO (4-amino-5-methylamino-2′,7′-difluorescein [DAF], 5 μmol/L, 5 min). Images were acquired using Olympus BX51 epifluorescence microscope. Fluorescence intensity was measured as previously described (Ryoo et al., 2008) using Metamorph software.

**Western blot and eNOS dimer blot analyses**

Cells were lysed in SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) and sonicated for 5 seconds to reduce sample viscosity. Each sample was resolved by 10% SDS-PAGE, transferred to PVDF membranes (Bio-Rad, Hercules, CA), analyzed with antibodies according to the supplier’s protocol, and visualized with peroxidase and enhanced chemiluminescence (Thermo Scientific, Rockford, IL). Samples were normalized to β-tubulin (1:1000, BD Bioscience, Franklin Lakes, NJ). Densitometry analysis of bands was performed using NIH ImageJ. Dimers and monomers of eNOS were separated by low-temperature SDS-PAGE and analyzed as described above (Ryoo et al., 2008).

**Vascular tension assay**

Male C57BL/6J wild type (WT) and HCD-fed ApoE−/− mice (D12108C, Research Diet Inc., USA) were anesthetized using isoflurane and the thoracic aortic vessel was rapidly removed. The aorta was placed in ice-cold oxygenated Krebs-Ringer bicarbonate solution (in mM;
NaCl 118.3, KCl 4.7, MgSO$_4$ 1.2, KH$_2$PO$_4$ 1.2, CaCl$_2$ 1.6, NaHCO$_3$ 25, glucose 11.1) and cleared of adherent connective tissues. The mouse aorta was cut into 1.5-mm rings and suspended between two wire stirrups (150 μm) in a myograph (Multi Myograph System DMT-620) containing 10 ml Krebs-Ringer (95% O$_2$-5% CO$_2$, pH 7.4, 37˚C). One stirrup was connected to a three-dimensional micromanipulator and the other to a force transducer. The rings were passively stretched at 10-minute intervals in increments of 100 mg to reach optimal tone (600 mg). After the arterial rings had been stretched to their optimal resting tone, the contractile response to 100 mM KCl was determined. The response to a maximal dose of KCl was used to normalize the responses to agonist across vessel rings. Dose responses to vasoconstrictors U46619 (10$^{-10}$-10$^{-6}$ M) and phenylephrine (PE, 10$^{-9}$-10$^{-5}$ M) were assessed, and responses to the vasodilators acetylcholine (Ach, 10$^{-9}$-10$^{-5}$ M) and sodium nitroprusside (SNP, 10$^{-9}$-10$^{-5}$ M) were assessed after preconstriction with U46619 (10$^{-8}$ M). To further confirm the vasorelaxation activity in a NO-dependent manner, vessels were treated with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase inhibitor. For measuring the effect of TDF on vascular smooth muscle cells, vessel rings were deendothelialized by gentle rubbing of the luminal surface (Supplemental Figure 1).

**Statistics**

Each graph represents cumulative data from three independent experiments performed in triplicate. Statistical significance was determined by one-way ANOVA (mean±SEM) with a post hoc test or t-test (mean±SEM) or by two-way ANOVA (mean±SD) (GraphPad Prism 5 software). A $P<0.05$ was considered statistically significant.
Results

TDF non-competitively inhibited arginase I and II activities in a concentration-dependent manner

TDF (Fig. 1) inhibited arginase I and II activities in a concentration-dependent manner (Fig. 2A and B, respectively). IC₅₀ values were 12.18 and 11.86 μM for arginase I and arginase II, respectively, and Eₘₐₓ at 20 μM TDF was 53.8% and 59.6% for arginase I and II, respectively. To test the biochemical property of TDF on arginase inhibition, we measured arginase activity prepared from liver and kidney lysates incubated at different concentrations of TDF (0, 5, and 10 μM) and L-arginine (0-50 μM) and performed Lineweaver-Burk plots. As shown in Fig. 2C and D, the slopes increased at higher concentrations of TDF (2.7 X 10⁻⁵ at 0 μM, 3.9 X 10⁻⁵ at 5 μM, 5.6 X 10⁻⁵ at 10 μM in Fig. 2C; 4.0 X 10⁻² at 0 μM, 6.2 X 10⁻² at 5 μM, and 1.49 X 10⁻¹ at 10 μM in Fig. 2D). The Y-intercepts were 3.3 X 10⁻⁶ with 0 μM, 4.3 X 10⁻⁶ with 5 μM, 4.6 X 10⁻⁶ with 10 μM TDF in Fig. 2C and 1.4 X 10⁻³ with 0 μM, 2.4 X 10⁻³ with 5 μM, and 4.0 X 10⁻³ with 10 μM TDF in Fig. 2D. These plots indicated that TDF was a non-competitive inhibitor of arginase I and II. A TDF concentration of 10 μM also inhibited arginase activity in both HUVECs (Fig. 2C) and aortic vessels (Fig. 2D) to a similar level as ABH, a known arginase inhibitor.

TDF-induced arginase inhibition reciprocally increased NO production and decreased ROS generation

In HUVECs, TDF incubation increased NO production in a dose-dependent manner (untreated 100±2.1% [12.4±0.26 μmol/mg protein], 110.3±2.5% with 5 μM TDF, and 119.2±3.2% with 10 μM TDF, Fig. 3A). In endothelium of aorta isolated from WT mice, TDF
treatment significantly increased the average slope of DAF fluorescence from 1.06±0.05 intensity/second in the untreated group to 1.56±0.07 intensity/second in the TDF-treated group, whereas the NOS inhibitor L-NAME reduced the slope to 0.17±0.04 intensity/second (Fig. 3B). Time-dependent fluorescence intensity of DHE was lower in TDF-treated aortas, with a DHE fluorescence slope of 0.25±0.04 intensity/second (versus 0.33±0.04 intensity/second in the untreated control), and was nearly quenched with MnTBAP (0.035±0.001 intensity/second) (Fig. 3C). Lucigenin chemiluminescence showed that xanthine reacted with xanthine oxidase to produce superoxide, indicating TDF was not acting as a ROS scavenger and did not affect the chemiluminescence signal (Fig. 3D).

**TDF induced eNOS phosphorylation and dimerization but did not change protein content in HUVECs**

As shown in Fig. 4A, TDF had no effect on the content of proteins, eNOS, or arginase I or II, and did not increase expression of inducible NOS (iNOS). However, TDF incubation significantly increased phosphorylation of eNOS at Ser1177 from 0.85 to 1.00 arbitrary units (AU) and resulted in a 36% increase in the dimer form of eNOS (Fig. 4B). We further tested that direct effect of TDF itself on eNOS dimerization by incubating cell lysates with TDF. However, TDF had no effect on eNOS dimerization (data not shown).

**TDF enhanced NO signaling in aortic vessels isolated from WT mice**

Because TDF augmented NO production in the aortic endothelium through enhanced eNOS phosphorylation and dimerization, we tested the effect of TDF on vessel reactivity in aortic vessels from WT mice. To confirm the effect of TDF on endothelium-dependent vasorelaxation, vessels were preconstricted with phenylephrine (PE, 5×10^-5 M), and dose-response curves of the endothelium-dependent vasodilator acetylcholine (Ach) and the
endothelium-independent NO donor sodium nitroprusside (SNP) were constructed. TDF incubation markedly accentuated Ach-induced maximal relaxation (\(E_{\text{max}}\), WT vs. WT+TDF, 84.0±1.8 vs. 104.2±1.7%, \(p<0.01\)) and decreased \(E_{50}\) (WT vs. WT+TDF, 1.2x10^{-7} vs. 2.3x10^{-8} M, \(p<0.01\), Fig. 5A). However, the response to SNP remained essentially unchanged in both groups (Fig. 5B, \(E_{\text{max}}\), WT vs. WT+TDF, 102.3±2.6 vs. 103.3±2.0%). The cumulative PE-dependent vasoconstrictor responses were significantly attenuated by TDF incubation (Fig. 5C, \(E_{\text{max}}\), WT vs. WT+TDF, 115.6±3.3 vs. 94.2±3.8%, \(p<0.01\); \(E_{50}\), WT vs. WT+TDF, 8.3x10^{-8} vs. 1.4x10^{-7} M, \(p<0.01\)). To further test whether the significant changes in vessels incubated with TDF were dependent on a mechanism of NO signaling, both groups of vessels were incubated with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) as an soluble guanylyl cyclase inhibitor. Incubation with ODQ further constricted both untreated and TDF-treated vessels, but the effect was not different between the groups of vessels (Fig. 5D WT vs. WT+TDF, 80.8±9.6 vs. 83.8±6.5%, not significant).

**TDF-dependent arginase inhibition increased NO production and decreased ROS generation in HCD-fed ApoE^{-/-} mice**

HCD-fed ApoE^{-/-} mice had higher total cholesterol (925±15.4 vs. 102±2.8 mg/dL), low-density lipoprotein (LDL, 275±8.7 vs. 33±3.5 mg/dL), and triglycerides (79.6±3.8 vs. 39.1±6.2 mg/dL) than did the WT mice fed a ND. HCD induced an increase in arginase activity (ApoE^{-/-}+HCD vs. WT+ND, 126.3±7.0 vs. 100±5.9%, *\(p<0.01\)) that was markedly decreased with TDF incubation (Fig. 6A, ApoE^{-/-}+HCD vs. ApoE^{-/-}+HCD+TDF, 126.3±7.0 vs. 94.1±2.9%, **\(p<0.01\)). TDF also improved HCD-induced impairments in endothelium-dependent NO production (Fig. 6B, WT+ND vs. ApoE^{-/-}+HCD, 1.06±0.05 vs. 0.29±0.05 fluorescence intensity/second, *\(p<0.01\), ApoE^{-/-}+HCD vs. ApoE^{-/-}+HCD+TDF, 0.29±0.05 vs. 0.75±0.03 fluorescence intensity/second, **\(p<0.01\)). However, TDF treatment reduced ROS production in
endothelium of HCD-fed ApoE\(^{-/-}\) mice (Fig. 6C, WT+ND vs. ApoE\(^{-/-}\)+HCD, 0.33±0.04 vs. 0.46±0.04 fluorescence intensity/second, \(p<0.01\), ApoE\(^{-/-}\)+HCD vs. ApoE\(^{-/-}\)+HCD+TDF, 0.46±0.04 vs. 0.32±0.04 fluorescence intensity/second, \(p<0.01\)). Interestingly, incubation with the NOS inhibitor L-NAME decreased ROS production in HCD-fed ApoE\(^{-/-}\) mice (ApoE\(^{-/-}\)+HCD vs. ApoE\(^{-/-}\)+HCD+L-NAME, 0.46±0.04 vs. 0.35±0.01 fluorescence intensity/second, \(p<0.01\)). This result suggested that endothelial NOS may be an important source of ROS production in endothelium of atherogenic aorta. Consistent with the results in HUVECs, TDF induced eNOS coupling (Fig. 6D) and phosphorylation at Ser1177 without changing eNOS protein content (Fig. 6E) in aortic vessels from HCD-fed ApoE\(^{-/-}\) mice.

**TDF improved endothelial dysfunction in ApoE\(^{-/-}\) mice fed a HCD**

Ach-dependent vasorelaxation was significantly attenuated in aortic rings from ApoE\(^{-/-}\) mice fed a HCD compared to those from WT fed a ND. The \(E_{\text{max}}\) was 56.7±2.7 vs. 95.3±1.7% (Fig 7A, ApoE\(^{-/-}\)+HCD vs. WT+ND, \(p<0.01\)). The reduced response to Ach markedly improved with TDF incubation, as shown by an \(E_{\text{max}}\) of 90.8±3.4% in the ApoE\(^{-/-}\)+HCD+TDF mice (\(p<0.01\)). However, the maximal response to SNP was comparable in all three groups (Fig. 7B, WT+ND, 98.6±2.6, ApoE\(^{-/-}\)+HCD, 94.7±1.6, ApoE\(^{-/-}\)+HCD+TDF, 93.4±4.7%). Furthermore, incubation with L-NAME induced vessel constriction to similar level in all groups (WT+ND, 132.9±17.2, ApoE\(^{-/-}\)+HCD, 124.5±16.5, ApoE\(^{-/-}\)+HCD+TDF, 126.8±10.9%, \(p<0.05\)), indicating that TDF enhanced eNOS-dependent NO signaling. However, the vasoconstriction response to U46619, a synthetic analog of the thromboxane A2 receptor agonist, increased in HCD-fed ApoE\(^{-/-}\) mice in a dose-dependent manner (Fig. 7C, WT+ND vs. ApoE\(^{-/-}\)+HCD, 331.4±8.3 vs. 440.3±20.2%, \(p<0.05\)). Interestingly, TDF preincubation of vessels from HCD-fed ApoE\(^{-/-}\) mice significantly decreased the maximal response to U46619 (ApoE\(^{-/-}\)+HCD, 440.3±20.2, ApoE\(^{-/-}\)+HCD+TDF, 318.5±14.5%, \(p<0.01\)). Inhibition of NOS with L-NAME
induced vessel constriction to a similar level in all groups (Fig 7D, WT, 132.9±17.1, ApoE−/− +HCD, 124.5±16.5, ApoE−/−+HCD+TDF, 126.8±10.9%).

Discussion

Arginase expressed in the vascular endothelium reciprocally regulates eNOS activity, which is associated with development of vascular diseases. Therefore, arginase inhibition may have a beneficial effect on vascular diseases associated with endothelial dysfunction by restoring eNOS activity. Here we demonstrated that TDF from Scutellaria indica was a dose-dependent, non-competitive inhibitor of arginase activity and increased NO production through eNOS coupling and phosphorylation at Ser1177. These TDF-mediated effects contributed to Ach-dependent vasorelaxation in WT and atherosclerosis-prone ApoE−/− mice fed a HCD. The inhibitory activity of TDF was comparable to that of ABH, a boronic acid derivative known to inhibit arginase.

NO has multiple vasoprotective characteristics, which has led researchers to investigate NO-based therapeutics that enhance NO signaling including dietary L-arginine (Blum et al., 2000; Boger, 2008; Dudek et al., 2004; Walker et al., 2001; Wilson et al., 2007), drug-eluting stents (Ansel and Lumsden, 2009), NOS gene therapy (Barbato et al., 2003; Kibbe and Tzeng, 2000), and NO inhalation (Griffiths and Evans, 2005; Ichinose et al., 2004). However, these strategies have also been associated with adverse effects such as increased genetic mutations, vascular injury, and systemic NO toxicity. In addition, clinical studies investigating an oral L-arginine supplement for treatment of vascular diseases has shown conflicting results, including worsened vascular diseases in three long-term studies (Dudek et al., 2004; Walker et al., 2001; Wilson et al., 2007). Currently however, most studies show arginase
inhibition restores endothelial dysfunction (Steppan et al., 2013). Therefore, TDF may be a good model for further development of an effective and safe novel therapeutic compound for atherosclerosis. The present study showed that TDF was a potent arginase inhibitor and improved endothelial dysfunction by attenuating the vasoconstriction response to PE and U46619, and augmenting the vasorelaxation response to Ach through by increasing NO bioavailability in normal (Fig. 5) and HCD-fed atherogenic-prone mice (Fig. 7).

Arginase activity reciprocally regulated eNOS-dependent NO production (Fig. 3) in the vascular endothelium. Increased arginase activity may reduce NO bioavailability in cardiovascular pathologies including vascular dysfunction (Berkowitz et al., 2003; Boger et al., 1995; Santhanam et al., 2007), asthma (Maarsingh et al., 2009), erectile dysfunction (Christianson, 2005), aging (Berkowitz et al., 2003), and atherosclerosis (Ryoo et al., 2008). The HCD increased serum LDL and activity of arginase II, which is the major arginase isoform in mouse aortic vessels (Ryoo et al., 2008), in ApoE−/− mice (Fig. 6A).

The protein content of eNOS increases in animal models of atherosclerosis (Kawashima, 2004). Relative to WT mice, eNOS-overexpressing mice have increases in HCD-induced atherosclerotic lesions whereas eNOS-deficient mice have decreases in lesions (Ozaki et al., 2002; Shi et al., 2002). As shown in Fig. 6, NO production decreased in HCD-fed ApoE−/− mice despite an increased abundance of eNOS protein, suggesting that coupling of eNOS activity and NO production is more important than increasing eNOS protein content. Furthermore, eNOS inhibition with L-NAME significantly decreased ROS production in HCD-fed ApoE−/− mice, suggesting that uncoupled eNOS is an important ROS-producing enzyme that contributes to atherosclerosis (Fig. 6D). Several mechanisms have been implicated in eNOS uncoupling under pathophysiological conditions, including: 1) L-arginine depletion, 2) cofactor depletion, 3) altered eNOS phosphorylation, and 4) protein-protein interactions.
among Ca2+/calmodulin, caveolin, and HSP90. Arginase inhibition has been shown to increase intracellular L-arginine concentration and stabilize the eNOS dimer (Woo et al., 2013). We showed that arginase inhibition with TDF induced eNOS phosphorylation at Ser1177 and increased the ratio of eNOS dimer/monomer (Fig. 3 and 6, respectively). A previous study showed arginase inhibition did not change BH4 content, total biopterin, or BH4/BH2 ratio (Ryoo et al., 2008). Therefore, arginase inhibition may have an alternative mechanism for eNOS coupling associated with phosphorylation that accompanies the increase in L-arginine concentration. Indeed, increased arginase activity with a high-fat diet (HFD) has been associated with p38 MAPK activation and subsequent eNOS uncoupling, whereas arginase II-null mice fed a HFD have reduced activation of p38 MAPK in the aorta, which protects them from eNOS uncoupling and endothelial dysfunction (Yu et al., 2014).

In conclusion, we showed that TDF non-competitively inhibited arginase activity and reciprocally increased NO production through increased eNOS phosphorylation and dimerization. TDF improved vascular function in both ND-fed WT and HCD-fed ApoE−/− mice. These results suggest that TDF may contribute to the improvement of vascular diseases caused by endothelial dysfunction.

**Authorship Contributions**

*Participated in research design:* J.H. Lee, B.S. Min, S. Ryoo.

*Conducted experiments:* H. M. Hwang, B. H. Jeon, S. Ryoo.

*Contributed new reagents or analytical tools:* B.S. Min, Y. M. Kim.

*Performed data analysis:* B. S. Min, Y. M. Kim, K. L. Hoe, S. Ryoo.
Wrote or contributed to the writing of the manuscript: S. Ryoo.

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Footnotes

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**Figure legends**

**Fig. 1.** Chemical structure of (2S)-5,2'5'-trihydroxy-7,8-dimethoxy flavanone (TDF).

**Fig. 2.** Inhibitory effect of TDF on arginase I and II activities.

Lysates from mouse liver and kidney were prepared in solutions containing arginase I (A) and II (B), respectively. Arginase activity was measured in the presence of the indicated concentration of TDF (one-way ANOVA, p<0.01). Lineweaver-Burk plots showed that TDF non-competitively inhibited arginase I (C) and II (D). HUVECs (E) and aortic vessels from WT mice (F) were treated with 10 μM TDF or ABH, a known arginase inhibitor, for 12 hours, and arginase activity was measured. * vs. untreated control, p<0.01 (t-test).

**Fig. 3.** TDF increased NO generation and decreased ROS production in endothelium.

HUVECs were incubated with TDF at different concentrations for 16 hours, and NO production was measured using Griess reagents (A, * vs. untreated control, p<0.05, n=4). (B) Aortic segments from WT mice were incubated with or without TDF (10 μM) for 12 hours. Time-dependent changes in fluorescence intensity of 4-amino-5-methylamino-2',7'-difluorescein (DAF, 5 μM) were measured using epifluorescence microscopy (endothelium side up). The slope of DAF fluorescence over 5 minutes increased significantly in TDF-incubated vessels (* vs. untreated, p<0.01, n=6). L-NAME (100 μM) was used as a control. (C) ROS production in endothelium of aortic vessels isolated from WT mice was determined using dihydroethidine (DHE, 1 μM), a superoxide-specific fluorescent dye. The slope was defined as the change in DHE fluorescence intensity over time. TDF incubation decreased the fluorescence slope (* vs. untreated, p<0.01, n=6). MnTBAP (10 μM), a superoxide scavenger used as a control, nearly eliminated the slope. (D) TDF did not affect xanthine
and xanthine oxidase-derived superoxide production. All statistical analyses were performed by t-test.

**Fig. 4.** TDF enhanced eNOS phosphorylation and dimerization in HUVECs.

(A) HUVECs were incubated with TDF (10 μM, 16 hours) and protein content was analyzed by western blot. TDF incubation increased eNOS phosphorylation at Ser1177 but did not change protein content (* vs. untreated, p<0.05, t-test). (B) Dimerized eNOS was separated using low-temperature SDS-PAGE and western blot analysis. TDF induced eNOS coupling (* vs. untreated, p<0.01, t-test). BC indicates boiling control. PC is a positive control for iNOS.

**Fig. 5.** TDF incubation increased NO signaling in aortic vessels from WT mice.

(A) TDF-treated vessels had increased endothelium-dependent vasorelaxation response to acetylcholine (Ach) (*, p<0.01, n=8). (B) Relaxation response to endothelium-independent relaxant SNP was not affected by TDF incubation.(C) Contractile responses to PE were attenuated in TDF-treated aortic rings (*, p<0.01, n=8). (D) PE-preconstricted aortic rings were further constricted with ODQ treatment (10^6 M) in both groups (* vs. untreated, p<0.01, t-test), but the responses were not different between TDF-treated and untreated groups.

**Fig. 6.** TDF-dependent arginase inhibition improved high cholesterol diet (HCD)-induced endothelial dysfunction in ApoE−/− mice.

(A) Increased arginase activity in aortas of HCD-fed ApoE−/− mice was attenuated by TDF incubation (10 μM) (* vs. WT+ND, p<0.01, ** vs. ApoE−/−+HCD, p<0.01, n=4). (B) TDF-induced arginase inhibition (10 μM) restored NO production in HCD-fed ApoE−/− mice (* vs. WT+ND, p<0.01, ** vs. ApoE−/−+HCD, p<0.01, n=6). L-NAME (100 μM) was used as a control. (C) HCD-induced increase in ROS production in ApoE−/− mice was reduced to the level of WT+ND (* vs. WT+ND, p<0.01, ** vs. ApoE−/−+HCD, p<0.01, n=6), and the NOS inhibitor L-NAME decreased the slope of DHE fluorescence (# vs. ApoE−/−+HCD, p<0.01, n=4).
MnTBAP was used as a ROS scavenger. (D) TDF augmented eNOS coupling in ApoE−/− mice fed a HCD (* vs. ApoE−/−+HCD, p<0.01, n=6). BC was a boiling control. (E) TDF incubation increased phosphorylation at Ser1177 but did not change eNOS protein content (* vs. ApoE−/−+HCD, p<0.01, n=4). All statistical analyses were performed by t-test.

Fig. 7. TDF restored HCD-induced impairment in vasoreactivity.

(A) Vessels were preconstricted to 60-75% of the contractile $E_{\text{max}}$ with U46619 (10⁻⁸ M), and cumulative dose-response curves to Ach was performed. The $E_{\text{max}}$ to the vasorelaxant Ach was markedly attenuated in rings isolated from HCD-fed ApoE−/− relative to those of ND-fed WT mice (WT+ND vs. ApoE−/−+HCD, *p<0.01, n=4, two-way ANOVA), whereas TDF incubation accentuated the vasorelaxant response in aortas from HCD-fed ApoE−/− mice (ApoE−/−+HCD vs. ApoE−/−+HCD+TDF, #p<0.01, n=4, two-way ANOVA). (B) Vasorelaxant responses to SNP were similar among all groups. (C) Maximal vasoconstriction ($E_{\text{max}}$) and $EC_{50}$ with U46619 (10⁻¹⁰ to 10⁻⁵ M) were significantly augmented in vessels from HCD-fed ApoE−/− mice relative to ND-fed WT mice (WT+ND vs. ApoE−/−+HCD, *p<0.01, n=4, two-way ANOVA), which was attenuated with preincubation of TDF (10 μM) (ApoE−/−+HCD vs. ApoE−/−+HCD+TDF, #p<0.01, n=4, two-way ANOVA). (D) The NOS inhibitor L-NAME (100 μM) induced vessel constriction to a similar level in all groups (* vs. untreated, p<0.01, n=4, t-test).
Figure 2-1
Figure 2-2

Relative arginase activity (%)

E
HUVECs

F
Aorta

Untreated
TDF (10 μM)
ABH (10 μM)

Untreated
TDF (10 μM)
ABH (10 μM)
Figure 3
**Figure 4**

A

- iNOS
- p-eNOS (Ser1177)
- eNOS
- Arg I
- Arg II
- Actin

TDF (μM) 0 10 PC

B

- TDF (μM) 0 BC 10 BC

Relative ratio of dimer/monomer

Protein amount/actin (AU)

TDF (10 μM) - + - + - + - +

- eNOS
- p-eNOS
- Arg I
- Arg II

*
Figure 5
Figure 6-1
Figure 6-2
Figure 7