Effect of Short-term Polyphenol Treatment on Endothelial Dysfunction and Thromboxane A_2 Levels in Streptozotocin-Induced Diabetic Mice

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Diabetes is characterized by the development of endothelial dysfunction, which affects both nitric oxide (NO)-mediated relaxation and endothelium-derived contracting factors, associated with vascular oxidative stress. There is a growing body of evidence suggesting that polyphenols have several beneficial effects, such as antioxidant and anti-inflammatory activities. This study investigated whether short-term treatment with polyphenols (chlorogenic acid (CA), morin (MO), resveratrol (RV)) can improve endothelial dysfunction related to diabetes. Aorta reactivity was determined in organ chambers, and we measured NO production and thromboxane B_2 (TXB_2; a metabolite of TXA_2) from aortas in response to acetylcholine (ACh). Streptozotocin (STZ)-induced diabetic mice (16 weeks) were injected with solvent (ethanol, 10% v/v; intraperitoneally (i.p.)), CA (0.03 mmol/kg/d), MO (0.03 mmol/kg/d), and RV (0.03 mmol/kg/d) for 5 d. The ACh-induced endothelium-dependent relaxation was markedly reduced in rings of STZ-induced diabetic mice compared to controls. The treatment with polyphenols (significantly: MO, tendency: CA and RV) for only 5 d improved the NO components of relaxation, but did not normalize ACh-stimulated NO production. However, polyphenol treatment suppressed the ACh-stimulated level of TXB_2 in aortas from STZ-induced diabetic mice. Thus, treatment with polyphenols caused basal NO production and a prompt improvement of the endothelial function in diabetic mice, and this may involve the normalization of TXA_2 levels, not NO production, under ACh stimulation.

Key words polyphenol; endothelial dysfunction; nitric oxide; thromboxane A_2

Diabetes mellitus is associated with increased cardiovascular morbidity and mortality *via* accelerated atherogenesis.[1,2] However, this excess cardiovascular risk cannot fully account for the traditional risk factors,[3] and it has been proposed that it may be at least partly associated with hyperglycemia.[4] Hyperglycemia itself is likely to increase the risk *via* a number of mechanisms that may encompass a combination of acute and chronic inflammation. An inflammation-centered model suggests that this risk may be driven by increased endothelial dysfunction.[5]

Endothelial dysfunction is a major event in the pathogenetic cascade leading to cardiovascular events,[4] and therapies aiming at preserving the endothelium are needed for the effective prevention of cardiovascular disease. The endothelium plays a central role in physiological maintenance of the vascular function by regulating vascular tone, leukocyte adhesion, and platelet activation, as a result of the release of vasoactive substances such as nitric oxide (NO), prostacyclin, and thromboxane A_2 (TXA_2).[1,6–16]

Several natural polyphenols have been evaluated for their ability to protect against endothelial dysfunction and, thus, for their effectiveness in preventing cardiovascular disease.[17] Indeed, we have also shown that long-term chlorogenic acid (CA) treatment (2 months) can normalize the impaired endothelium-dependent relaxation seen in diabetic rats.[18] Although these effects of polyphenol treatment could be secondary to its protective effect against oxidant stress, there is preliminary evidence indicating that polyphenols themselves may contribute to regulation of the vascular tone.[19] When administered *in vitro*, resveratrol (RV) enhances endothelial vasorelaxation by potentiating NO synthase and increasing the phosphorylation of Akt and endothelial nitric oxide synthase (eNOS), suggesting that RV alone has a vasodilatory effect.[19] We hypothesized that short-term treatment with polyphenols improves endothelial dysfunction related to diabetes.

Here, we assessed the effects of common, dietary, natural polyphenols in diabetic mice. We selected the common dietary polyphenols of CA (3-(3,4-dihydroxycinnamoyl) quinate), morin (MO: 3,5,7,2′,4′-pentahydroxyflavone), and RV (3,5,4′-trihydroxystilbene) for this study. CA is a widely distributed phenolic acid present in coffee and some fruits, and it is known as a water-soluble antioxidant, and it has been successfully encapsulated in low-cost, high-volume yeast cells for the first time.[20–23] MO is a flavonoid found in figs and other Moraceae, which are used as herbal medicines, and it is known as a major lipid-soluble antioxidant.[22–24] RV is a naturally occurring polyphenolic phytoalexin found in many plants and contained in foods and drinks such as in Mediterranean diets and French wine, and it is known as a poor water-soluble antioxidant.[25,26] Regarding concerns about the bioavailability of polyphenols, we examined the effects of short-term treatment with each polyphenol. Furthermore, whether or not short-term treatment with polyphenols can improve endothelial dysfunction, impaired NO production, and increased endothelium-derived contraction factors related to diabetes is unknown. From these viewpoints, we examined whether these polyphenols would have an effect.

In the present study, we investigated the effects of short-term polyphenol treatment involving, CA, MO, and RV on the endothelium-dependent relaxation of aortic rings isolated from streptozotocin (STZ)-induced diabetic mice, compared the three polyphenols, and clarified some of the underlying

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molecular mechanisms. To investigate such aspects, diabetic mice were treated with each polyphenol for only 5 d.

MATERIALS AND METHODS

Reagents MO was purchased from Kanto Chemical (Tokyo, Japan). CA was purchased from Cayman Chemical Company (Michigan, U.S.A.). RV was purchased from Tokyo Chemical Industry (Tokyo, Japan). STZ was purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Sodium nitroprusside (SNP) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), while acetylcholine (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). MO and RV were dissolved in ethanol, and then a specified amount of distilled water was added and they were mixed. All other agents were dissolved in saline. All concentrations are expressed as the final molar concentration of the base in the organ bath.

Animals and Experimental Design Male Institute of Cancer Research (ICR) control mice (5 weeks old, at Tokyo Animal Laboratories, Tokyo, Japan) were divided randomly into five experimental groups; Group I: Nondiabetic mice used as a control group (n=6); Group II: STZ-induced diabetic group (n=6); Group III: STZ-induced and CA-treated diabetic group (n=6); Group IV: STZ-induced and MO-treated diabetic group (n=6); Group V: STZ-induced and RV-treated diabetic group (n=6). Diabetes was induced by an injection *via* the tail vein of STZ (200 mg/kg) dissolved in citrate buffer (Groups II–V).[27,28] Age-matched control mice were injected with the buffer alone (Group I). Food and water were available *ad libitum*. The experiments described here were performed 11–12 weeks after the injection. The mice were euthanized with inhaled isoflurane and assigned to various experiments. The animal protocols were approved as conforming to the Guide for the Care and Use of Laboratory Animals by the issuing committee (the Committee on the Care and Use of Laboratory Animals of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan).

CA, MO, and RV Treatments Ten percent ethanol as a vehicle was injected into Group II. No inflammation was observed at the intraperitoneally (i.p.) injected site of mice. The mice in Group III (STZ-induced and CA-treated diabetic group) were i.p. injected with CA (0.03 mmol/kg/d) for 5 d. The mice in Group IV (STZ-induced and MO-treated diabetic group) were i.p. injected with MO (0.03 mmol/kg/d) for 5 d. The mice in Group V (STZ-induced and RV-treated diabetic group) were i.p. injected with RV (0.03 mmol/kg/d) for 5 d. In this study, we examined cumulative concentration–response curves for CA, RV, and MO (10^{-9}–10^{-5} mol/L) (data not shown). Moreover, Shin *et al.* reported that intraperitoneally administered CA (10 mg/kg/d) had a protective effect against diabetic retinopathy in a diabetic rat model.[29] Considering that CA 10 mg/kg is equivalent to CA 0.03 mmol/kg, this concentration (CA, RV, and MO: 0.03 mmol/kg/d) was selected.

Measurement of Plasma Parameters Plasma parameters were measured as described previously.[8–16,27,28] Plasma samples were stored at −20°C until analysis. Briefly, plasma glucose, cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, and serum non-esterified fatty acid (NEFA) levels were each determined with a commercially available enzyme kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Measurement of Isometric Force Measurement of the isometric force was performed as described previously.[15,16,27] Briefly, the thoracic aortas were carefully isolated from mice, dissected from surrounding fat and connective tissue, cut into circular segments (2 mm long), and immediately placed in Krebs–Henseleit Solution (KHS), concentration in mmol/L: NaCl, 118.0; KCl, 4.7; $NaHCO_3$, 25.0; $CaCl_2$, 1.8; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 11.0. Aortic rings were suspended in 10 mL jacketed organ baths filled with 10 mL of KHS continuously aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C. The rings were equilibrated for 45 min under a resting tension of 1.5 g before the experiment. During the equilibration period, the rings were washed every 15 min. At the end of the equilibration, rings were contracted with a submaximal concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-6}–3×10^{-6} mol/L), the induction of stable and reproducible contractions in each aortic preparation was confirmed by comparing them with the first contraction induced using 80 mM K^+, with the latter being taken as 100%, which produced 70–80% of the maximal response. After reaching a plateau of contraction, cumulative concentration-response curves to ACh (10^{-9}–10^{-5} mol/L) and sodium nitroprusside (SNP; 10^{-10}–10^{-5} mol/L) were obtained for relaxations.

Measurement of NO Production NO detection (nitrite+nitrate) was sampled and performed as previously described.[14] The collected blood samples were immediately heparinized and then centrifuged at 2000 rpm for 10 min at 4°C to separate the plasma. The separated plasma was mixed with an equivolume of methanol and centrifuged (13000 rpm, 4°C for 10 min) to obtain supernatant for plasma NOx measurements. Each aorta was cut into transverse rings of 4 mm in length. These were placed in KHS at 37°C and then treated with ACh (10^{-6} mol/L) for 15 min. NOx (nitrite+nitrate) was measured with the HPLC/Gries system (ENO-20, Eicom, Kyoto, Japan).

Measurement of TXB_2 Levels in Aortas Each aortic ring was placed for 10 min in a siliconized tube containing KHS at 37°C, and then 10^{-6} mol/L of ACh or vehicle (water) was applied for 15 min. Next, after the thoracic aortic rings had been removed, the tubes were freeze-clamped in liquid nitrogen and stored at −80°C for subsequent analysis.

Thromboxane release was measured as in our previous studies.[2] TXB_2, a metabolite of TXA_2, was measured using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). The amount of TXB_2 is expressed in pictograms per milligram wet weight of the aortas.

Data Analysis Experimental vasorelaxation values are expressed as a percentage of the maximal contraction induced by $PGF_{2\alpha}$ in a given segment. Concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 6.0; GraphPad Software, San Diego, CA, U.S.A.). Data are expressed as the mean±S.E., and n represents the number of mice. Statistical analysis was performed by one- or two-way ANOVA, with ANOVA for repeated measures and subsequent Bonferroni *post-hoc* tests. The p-values less than 0.05 were considered significant.

RESULTS

Characteristics of STZ-Induced Diabetic Rats At the time of the experiment, the body weight was significantly

Table 1. Change in Weight Gain of STZ-Induced Diabetic Mice and Those Treated with Chlorogenic Acid (CA), Morin (MO), or Resveratrol (RV)

	STZ	STZ-CA	STZ-MO	STZ-RV
Weight gain (g/5 d)	1.3±1.3	0.3±0.5	2.0±0.5	1.2±2.0

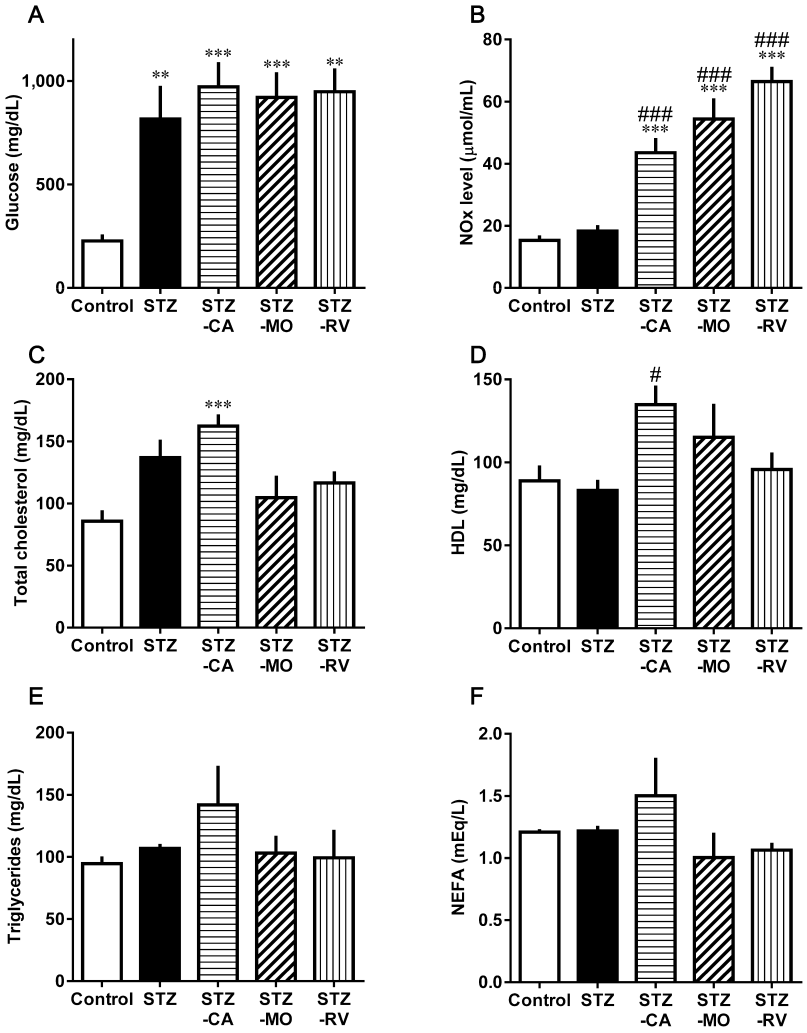

Fig. 1. Characteristics of Control, STZ-Induced Diabetic, and STZ-Induced Diabetic Mice Treated with Polyphenols (CA, MO, and RV)

Plasma glucose (A), plasma NO production (B), total cholesterol (C), HDL (D), triglyceride (E), and serum NEFA (F) levels were measured in control, STZ, STZ-CA, STZ-MO, and STZ-RV (n=6) mice. Values are means±S.E. $^{**}p<0.01$ or $^{***}p<0.001$ *vs.* Control, $^{\#}p<0.05$ or $^{\#\#\#}p<0.001$ *vs.* STZ.

lower in STZ-induced diabetic ($p<0.001$)(30.1±2.1 g, n=6) than control (49.2±2.2 g, n=6) mice. Table 1 shows the change in weight gain between pre- and after-treatment with the polyphenols (CA, MO, and RV). There were no differences in the body weight change among STZ-induced diabetic, STZ-induced and CA-treated diabetic, STZ-induced and MO-treated diabetic, and STZ-induced and RV-treated diabetic mice during the entire experimental period.

Figure 1 shows plasma glucose, NO production, total cholesterol, triglyceride, and NEFA levels at the end of the experiment. Plasma glucose was significantly higher in STZ-induced diabetic than control mice (Fig. 1A). The polyphenol treatments did not affect the glucose levels of STZ-induced diabetic mice. To determine whether the polyphenols (CA, MO, and RV) increase NO production in diabetes, NO levels were determined by measuring NOx (NO_2+NO_3). In STZ-induced diabetic mice, treatment with CA, MO, and RV significantly increased NO production, suggesting that polyphenols stimulate its production (Fig. 1B). Although the final total cholesterol levels of the CA-treated group were higher than those of the control, there was no difference between the other groups and controls (Fig. 1C). Also, although HDL levels of the CA-treated group were higher than those of STZ-induced diabetic mice, there were no differences between the other groups and STZ-induced diabetic mice (Fig. 1D). Triglycerides and NEFA were not different among the five experimental groups (Figs. 1E, F).

Relaxation Responses to ACh and SNP Endothelium-dependent relaxation of aortic rings precontracted with $PGF_{2\alpha}$ in response to ACh is summarized in Fig. 2A. ACh-induced relaxations in STZ-induced diabetic mice were decreased compared with controls. The impaired relaxation in response to ACh in aortic rings from diabetic mice reached near control levels within 5 d of MO treatment (STZ-MO). MO treatment

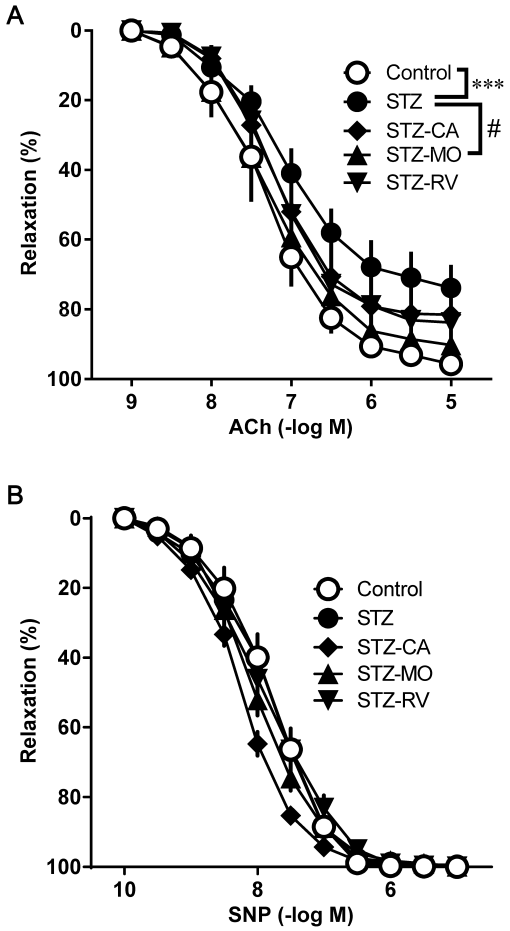

Fig. 2. Endothelium-Dependent Relaxation in Response to ACh (A) and Endothelium-Independent Relaxation in Response to SNP (B) in the Aorta

Values are means±S.E. ***$p<0.001$ *vs.* Control, $^{\#}p<0.05$ *vs.* STZ.

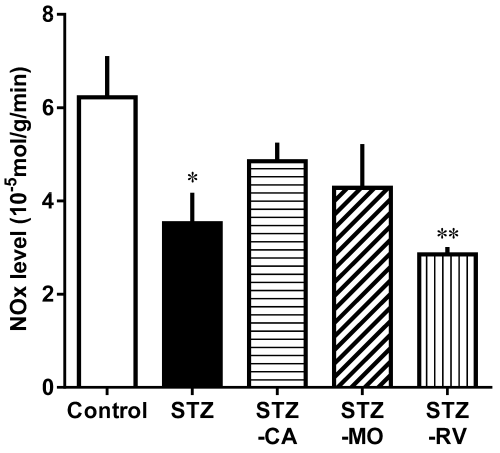

Fig. 3. Release of Tissue NOx under ACh Stimulation in Aortic Rings

The release of NOx in aortic rings from control, STZ-induced diabetic, and STZ-induced diabetic mice treated with CA, MO, or RV. Aortic samples underwent ACh (10^{-6}M; 15 min) stimulation. Values are means±S.E. *$p<0.05$ or **$p<0.01$ *vs.* Control.

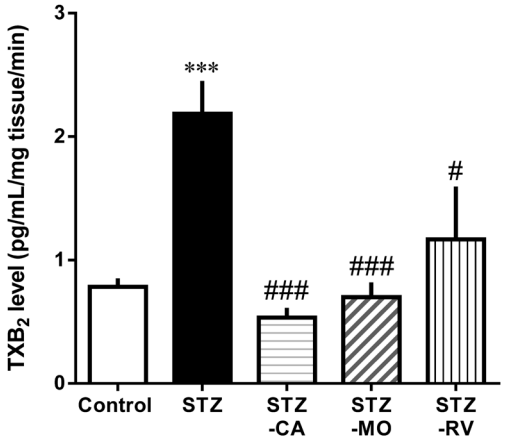

Fig. 4. Release of TXB_2 (a Stable Metabolite of TXA_2) under ACh Stimulation in Aortic Rings

The release of TXB_2 in each polyphenol-treated or untreated aortic ring from control or STZ-induced diabetic mice with ACh (10^{-6}M, 15 min) stimulation. Values are means±S.E. ***$p<0.001$ *vs.* Control, $^{\#}p<0.05$ or $^{\#\#\#}p<0.001$ *vs.* STZ.

significantly ameliorated ACh-induced relaxations in diabetic mice. Although the impaired relaxation in response to ACh in aortic rings from CA-treated and RV-treated mice showed a trend toward an increase compared with the control, the differences did not reach significance.

Endothelium-independent relaxations in response to SNP in aortic rings precontracted with $PGF_{2\alpha}$ were not significant when compared to control and STZ-induced diabetic groups (untreated, CA-treated, MO-treated, and RV-treated). Data were shown in Fig. 2B.

Polyphenols Stimulates NO Production ACh-induced NO productions were determined in aortic tissues obtained from each group. ACh-stimulated NO production was decreased in aortic rings isolated from STZ-induced diabetic mice (Fig. 3). However, despite our expectation, the STZ-induced diabetic mice treated with the polyphenols (CA, MO, and RV) did not show improved ACh-stimulated NO production, demonstrating the importance of other signaling pathways in NO regulation.

Polyphenols (CA, MO, and RV) Decrease TXA_2 in Aortas To investigate what the other signaling is, we assessed the production of TXB_2, a metabolite of TXA_2, stimulated with ACh (10^{-6}mol/L) in aortic rings from control, STZ, STZ-CA, STZ-MO, and STZ-RV (Fig. 4). The levels of TXB_2 release stimulated by ACh increased in the STZ group. However, in the STZ mice treated with CA, MO, and RV, the TXB_2 levels were much lower than those observed in STZ mice.

DISCUSSION

This is the first study showing an acute protective effect of polyphenols on endothelial function with a concurrent reduction in an endothelium-derived contracting factor (TXA_2) in diabetic mice. Furthermore, we show that polyphenols activate NO production.

Polyphenols have been shown to exhibit multiple potential health benefits, including cardiovascular protection.[30,31] Although the underlying mechanisms remain to be elucidated, evidence suggests that they may be related to the antioxidant and anti-inflammatory properties of polyphenols and their ability to improve endothelial function.[30] Particularly, RV can relax isolated murine aortas or porcine mesenteric and uterine arteries.[32] It appears that NO-mediated pathways are involved in RV-induced vascular relaxation.[33] In the present study, CA, MO, and RV treatment markedly increased NO produc-

tion in diabetes. This strongly suggests that the polyphenols themselves increase NO production. The observation that the polyphenols increased NO-mediated processes after only 5 d suggests that they can acutely activate NO-mediated processes to increase NO production. Furthermore, we thought that polyphenols might control NO degradation, because only 5-d treatments with polyphenols maintained a very high concentration of NO. However, the mechanisms by which polyphenols act on diabetic mice to cause NO production and control NO degradation will require further investigation by studying responses in mice.

Endothelial dysfunction is an early event in the development of atherosclerosis, and is closely associated with insulin resistance and diabetes. Many factors are associated with endothelial dysfunction, including chronic inflammation. As above, polyphenol treatment has been shown to improve endothelial function in hypertensive rats,[34] in rats fed a high-fat diet,[35] and diabetic mice.[36] This is not surprising since polyphenols have been shown to exhibit potent antioxidant and anti-inflammatory properties.[36] Our observation of impaired endothelium-dependent vasorelaxation in response to ACh in diabetic mice is consistent with previous reports.[2,10,11,37] Moreover, we found that endothelium-dependent vasorelaxation of isolated aortas in response to ACh was improved by MO in diabetic mice and showed a marked tendency to improve by CA and RV; because the polyphenols reduced TXA_2 levels in the aorta, the subsequent reduction of contraction factors may, at least in part, account for the observed improvement of endothelium-dependent vasorelaxation. That is, the polyphenols did not modify ACh-stimulated NO production, but suppressed TXA_2 levels in the diabetic aorta. We found that the polyphenols restored the balance between the vasorelaxation factor and contraction factor in the endothelium (because there was no change in the response to SNP), and increased sensitivity to basal NO in diabetic aortas. In addition, polyphenols may decrease TXA_2 levels *via* the suppression of reactive oxygen species and oxidative stress production in the aortas, but we have no detailed data on the mechanisms. Regarding these points, further study is needed.

Finally, there may be differences among the 3 polyphenols because many polyphenols can act as antioxidants in chemical systems and food matrices, as their extensive conjugated π-electron systems facilitate the donation of electrons from hydroxyl moieties to oxidizing radical species,[38] and phenolic compounds are a class of lipid-soluble chain-breaking antioxidants and highly effective water-soluble antioxidants in living organisms.[21] For example, CA is a water-soluble antioxidant.[21–23] MO is a lipid-soluble antioxidant but it is weak, and a very wide range of biological actions of MO, including inhibitory activity against, oxidative modification of low-density lipoprotein[22] and a vasorelaxant effect,[23] have been reported. *trans*-RV in plasma is very sparse and shows a short half-life (8–14 min) because of its poor water-solubility and instability, as it converts to a cis form (a less active form).[26] However, in this study, there were no significant differences among the 3 polyphenols. These results suggest that there may be no change in the biological effects of the absorbed polyphenols in the vascular system.

In conclusion, the results of this study demonstrate that polyphenols promote NO production and prompt improvement of the endothelial function in diabetic mice. These effects may be associated with the suppression of TXA_2 levels, but not the acceleration of NO production under ACh stimulation, in thoracic aortas from diabetic mice.

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