Resveratrol Ameliorates Clonidine-Induced Endothelium-Dependent Relaxation Involving Akt and Endothelial Nitric Oxide Synthase Regulation in Type 2 Diabetic Mice

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Diabetic vascular complication is one of the manifestations of endothelial dysfunction. Resveratrol (RV) is considered to be beneficial in protecting endothelial function. However, the exact protective effect and mechanisms involved have not been fully clarified. In this study, we investigated the relationship between Akt/endothelial nitric oxide synthase (eNOS) activation and RV in diabetes-induced endothelial dysfunction. Aortas were dissected and placed in organ chambers, and nitric oxide (NO) production in response to acetylcholine (ACh) and RV was measured. ACh-induced endothelium-dependent relaxation was markedly increased in controls by RV pretreatment. Furthermore, RV caused NO-dependent relaxation via the Akt signaling pathway, which was weaker in the aortas of diabetic mice than age-matched controls. To further examine the underlying mechanisms, we measured the phosphorylation of Akt and eNOS by Western blotting. RV caused the phosphorylation of Akt and eNOS in aortas, which was decreased in diabetic mice. However, RV augmented the impaired clonidine-induced relaxation in diabetic mice. Interestingly, the phosphorylation of Akt and eNOS was increased under stimulation with RV and clonidine only in diabetic mice. Thus, either RV or clonidine causes Akt-dependent NO-mediated relaxation, which is weaker in diabetic mice than controls. However, additional exposure to RV and clonidine has an augmenting effect on the Akt/eNOS signaling pathway under diabetic conditions. RV-induced Akt/eNOS activity may be a common link involved in the clonidine-induced Akt/eNOS activity, so RV and clonidine may have a synergistic effect.

Key words resveratrol (RV); endothelial dysfunction; nitric oxide (NO); Akt

Type 2 diabetes (DM) is associated with several complications, especially vascular complications (e.g., coronary insufficiency, cerebrovascular and peripheral vascular disease), which are the main etiologies leading to morbidity and mortality. Patients with DM exhibit complex vascular changes, such as an accelerated atherosclerosis process and hypercoagulability. Atherosclerosis is a major factor that accelerates diabetic vascular complications, and vascular endothelial dysfunction is considered to be the earliest detectable abnormality in the process of atherosclerosis. Thus, improving vascular endothelial dysfunction plays an important role in diabetic treatment.

Resveratrol (RV; 3,5,4′-trihydroxystilbene) is a naturally occurring polyphenolic phytoalexin found in many plants, and it has been shown to prolong the lifespan of lower organisms. Foods and drinks rich in RV, such as Mediterranean diets and French wine, are associated with a reduced risk of cardiovascular mortality in humans, and evidence has confirmed that RV has antiapoptotic, anti-inflammatory, anti-aging, and anticancer effects as well as a cardiovascular protective effect. We previously reported that RV increased nitric oxide (NO) production in diabetic mice. However, the underlying mechanisms are complicated and remain unclear.

Endothelial dysfunction is characterized by decreased vasoprotective endothelial NO bioavailability resulting from numerous mechanisms. NO is an important signaling molecule in the control of vascular permeability, and endothelial NO synthase (eNOS) is a major source of NO. Although eNOS and NO are considered to be major players in endothelial function, studies have yielded controversial results about the effect of RV on them. As shown by previously released data, RV is also vasoactive and has been shown to cause vasodilation and improve aortic endothelial function in diabetic mice. In diabetic mice, RV preserved eNOS phosphorylation. However, the consumption of RV failed to affect vascular reactivity and NO production in DM patients. In addition, the major upstream effectors of the eNOS pathway include Akt. Evidence from our previous experimental data indicates that clonidine promotes the Akt/eNOS pathway, but it inhibits the pathway in the diabetic aorta. Furthermore, Xia et al. showed that RV increased Akt phosphorylation in endothelial progenitor cells. Given the importance of vasoprotective NO bioavailability in endothelial cells, the aim of this study was to determine the role of eNOS activity in RV-induced relaxation in aortic rings from control and diabetic mice. Specifically we investigated the relationship between RV-induced eNOS activity and clonidine-induced activity and the ameliorative Akt/eNOS activation in aortic rings from control and diabetic mice.

MATERIALS AND METHODS

Reagents Nicotinamide, streptozotocin (STZ), α-N(ω-nitro-l-arginine (L-NNA), clonidine, and monoclonal β-actin antibody were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Akt-inhibitor (IL-6-hydroxymethyl-chiro-inositol 2[(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate]) was purchased from CALBIOCHEM (San Diego, CA, U.S.A.), while acetylcholine (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). Sodium nitroprusside (SNP) was from Wako

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The isometric force was performed as described previously. The aortic rings were cut into 5-mm segments and put in KHS (37°C). Some samples were treated with Akt-inhibitor (10⁻⁶ mol/L) for 30 min, and then, all samples were exposed to RV (10⁻⁶ mol/L). Twenty minutes after RV treatment, the solution was sampled.

**Western Blotting** Protein expressions were measured as described previously. Each aortic ring was placed for 10 min in a siliconized tube containing KHS at 37°C, and then 10⁻⁶ mol/L of RV was applied for 15 min. Some samples were treated with clonidine (10⁻⁶ mol/L, for 20 min) under RV-stimulation (10⁻⁶ mol/L, for 30 min) or only RV (10⁻⁶ mol/L). Next, after the aortic rings had been removed, the tubes were freeze-clamped in liquid nitrogen and stored at −80°C for subsequent analysis. Frozen aortas were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mmol/L Tris–HCl (pH 7.6), 150 mmol/L NaCl, 1 w/v% Nonidet P-40, 1 w/v% sodium deoxycholate, and 0.1 w/v% sodium dodecyl sulfate (SDS); ThermoScientific, Rockford, IN, U.S.A.) containing protease and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, U.S.A.). The protein concentration of the samples was determined using the bicinchoninic acid protein assay reagent kit (Pierce). Protein samples (25 μg/ lane) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel. Separated bands were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. After blocking the residual protein sites on the membranes using a blocking solution (ImmunoBlock; DS Pharma Biomedical, Osaka, Japan), they were incubated with an anti-Akt antibody (1:1000), anti-phosphorylated Akt-Ser473 antibody (1:1000), anti-phosphorylated eNOS-Ser1177 antibody (1:1000), and anti-eNOS antibody (1:1000), anti-β-actin antibody (1:5000) in blocking solution overnight at 4°C. β-Actin was used as the protein loading control. After washing, the membrane was incubated with HRP-linked secondary antibodies (1:10000) for 20 min. Specific bands were detected using a SuperSignal (Thermo Fisher Scientific Inc., U.S.A.) with an enhanced chemiluminescence substrate for horseradish peroxidase, according to the manufacturer’s instructions, and quantified by densitometry. This protocol was previously described.

**Data Analysis** All data are expressed as the mean±standard error (S.E.), and analyzed using one- or two-way ANOVA with a post-hoc Bonferroni’s multiple comparisons test or Student’s t-test. p-Values less than 0.05 were considered significant. The relaxation response is expressed as a percentage of the contraction induced by PGF₂α. The aortic rings were precontracted with an equieffective contraction of prostaglandin F₂α (PGF₂α, 10⁻⁶–3×10⁻⁶ mol/L) and the induction of stable and reproducible contractions of each aortic preparation were confirmed by comparing them with the first contraction induced using 80 mmol/L K⁺, and the relaxant responses in each ring were determined based on the percentage reduction of the PGE₂-induced maximum contraction, the latter being taken as 100%. In the present study, the tension developed in response to 10⁻⁶–3×10⁻⁶ mol/L PGF₂α did not differ significantly between controls and DM mice. All figures were created with Graph Pad prism 6.0 (GraphPad Software Inc., San Diego, CA, U.S.A.).
RESULTS

Body Weight and Blood Glucose At the time of the experiment (when the mice were 17 weeks old), there were no differences in body weight change among the nicotinamide+STZ-induced diabetic mice (53.2±0.5 g; n=15) and age-matched nondiabetic control mice (54.3±1.0 g; n=15). All nicotinamide+STZ-induced diabetic mice exhibited hyperglycemia (771.9±84.8 mg/dL; n=15), with their blood glucose levels being significantly higher than those of the controls (151.9±10.6 mg/dL; n=15, p<0.001).

Influence of RV on Endothelium-Dependent and -Independent Relaxation We previously reported that RV generated abundant NO.8) We then studied the effects of RV using aortic ring preparations from controls. We performed 2 sequential experiments to construct the concentration–response curves (1st experiment: control, 2nd experiment: RV pretreatment). RV was used for pretreatment (for 30 min) before the PGF2α-induced pre-contraction. We confirmed that control sequential experiments performed after 30 min without RV showed the same contraction profile as the preceding control. The endothelium-dependent relaxations in response to ACh caused concentration-dependent relaxations in aortic rings, which were also greater in aortic rings from RV (10−6 mol/L; 30 min)-pretreated controls than non-pretreated controls, whereas endothelium-independent relaxations in response to SNP, a donor of NO, were similar in RV-pretreated controls and non-pretreated controls (Figs. 1A, B). The results suggest that RV may generate NO in the endothelium of mouse aortas.

RV caused vascular relaxations of aortas, which were abolished in the presence of L-NNA (10−4 mol/L), a competitive inhibitor of NO synthase, and in the presence of Akt inhibitor (10−6 mol/L) in aortic rings from controls, indicating that they are mediated exclusively by NO via the Akt signaling pathway in the endothelium (Fig. 1C).

Effect of RV in Diabetic Mice As shown in Fig. 2A, diabetic aortas showed significantly impaired endothelium-dependent responses in the presence of RV. Then, we analyzed NO production to ascertain the effect of RV (Fig. 2B). NO production without RV stimulation (as the basal condition) was similar in both Control and DM aortas (data not shown). NO levels decreased in the diabetic aortas under RV stimulation (Fig. 2B). Moreover, NO levels were significantly lower in the control aortas pretreated with Akt inhibitor under RV stimulation (Fig. 2B). From these results, we hypothesized that the controls may show elevated Akt-derived NO production after RV stimulation, and this response may decrease under diabetic conditions. So, diabetic mice and controls were assessed by Western blot analysis (Fig. 3A). Although RV did not affect the total amount of Akt between diabetic mice and controls, Akt phosphorylation was more markedly decreased in diabetic mice than controls (Figs. 3B, C). We subsequently investigated whether RV would induce the phosphorylation of eNOS, a downstream effector of Akt. Although total eNOS

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Fig. 1. Concentration–Response Curves for Drug-Induced Relaxation in the Aorta

(A) ACh, (B) SNP, and (C) RV-induced relaxation. Closed circle data represent treatment with RV (10−6 mol/L), Akt-inhibitor (10−6 mol/L), or L-NNA (10−4 mol/L) for 30 min before the contraction with PGF2α. Values are the mean±S.E., n=4–6, *p<0.05, **p<0.01, ***p<0.001 vs. Control.
protein expression was comparable between diabetic and control aortas, eNOS phosphorylation was decreased in diabetic aortas under RV stimulation (Figs. 3D, E).

**Relationship between RV and Clonidine in Diabetic Aortas**  The direct action of RV on the aortic ring was evaluated, as shown in Figs. 1–3. Additionally, clonidine, focused on in this study, is an $\alpha_2$-adrenoceptor agonist. Previously, we reported that the mechanism of clonidine-induced endothelium-dependent relaxation was via the Akt/eNOS signaling pathway and, in diabetes, the impaired clonidine-induced response was determined to be a cause of Akt/eNOS downregulation. Briefly, in this study, we hypothesized that RV and clonidine may have a synergistic effect on Akt/eNOS activation. So, the experiment was designed to investigate the mechanism of vasorelaxation induced by clonidine under RV stimulation in the diabetic and control aortas. As shown in Fig. 4A, clonidine induced a concentration-dependent relaxation in controls. Interestingly, the pre-incubation of aortic rings with RV reduced the response to clonidine. These results suggest that the effect of RV to inhibit vascular relaxation has the potential to inhibit
Fig. 4. Role of RV in Clonidine-Induced Relaxations in Aortas from Control (A) and DM (B) Mice
Aortic rings were treated with RV (10^{-6} mol/L) for 30 min before the contraction with PGF$_{2\alpha}$. Values are the mean ± S.E., n=5, ***p<0.001 vs. Controls, #p<0.05 vs. DM.

Fig. 5. Akt/eNOS Activations under RV or Clonidine Stimulation in Aortas from Control and DM Mice
(A) Representative Western blots. (B) Total Akt, (C) total eNOS, (D) Akt phosphorylation at Ser473, and (E) eNOS phosphorylation at Ser1177 expressions in aortas. Ratios were calculated for the optical density of Akt or eNOS over that of β-actin. Those phosphorylations were normalized by the total protein. Some samples were stimulated with RV (10^{-6} M) or clonidine (10^{-6} M) for 20 min. Values are the mean ± S.E., n=4, ***p<0.001 vs. vehicle Control, ’p<0.05 vs. RV-stimulated Control, $$$p<0.01 or $$$p<0.001 vs. clonidine-stimulated Control.
the activations of Akt and eNOS, leading to the suppression of NO production in control aortas. Meanwhile, in the diabetic mice, the relaxation in response to clonidine was inhibited, as shown in Fig. 4B. The relaxation response to clonidine was significantly increased by RV compared to the corresponding controls. These results suggest that the effect of RV on vascular relaxation may be, at least partially, mediated through clonidine-induced Akt and eNOS activation.

**Effect of Clonidine on Phosphorylation Levels of Akt and eNOS Proteins under RV Stimulation** In view of the published evidence, clonidine-induced Akt phosphorylation at Ser473 and eNOS phosphorylation at Ser1177 are significantly decreased in the diabetic aorta.14–18) So, we performed analysis of Akt phosphorylation at Ser473 and eNOS phosphorylation at Ser1177 during single stimulation with RV or clonidine in aortas from Control and DM mice (Fig. 5A). Total protein levels of Akt and eNOS under RV or clonidine stimulation were not altered in DM or Control mice (Figs. 5B, C). RV (10^-6 M) or clonidine (10^-6 M) -stimulated Akt phosphorylation was greater in the control than vehicle control (Fig. 5D). Furthermore, clonidine-stimulated eNOS phosphorylation was greater and RV-stimulated eNOS phosphorylation demonstrated an upward trend in the control compared to vehicle control (Fig. 5E). In contrast, these phosphorylations did not show a significant difference among vehicle, RV-stimulation, and clonidine-stimulation in the aortas from DM mice (but, eNOS phosphorylation tended to be greater in RV-stimulated aortas from DM than vehicle DM control) (Figs. 5D, E). In addition, those phosphorylations were greater under clonidine stimulation in the aortas from Control than those from DM mice (Figs. 5D, E). These data are consistent with the published evidence (about clonidine stimulation)14–18) and RV-stimulation data (Figs. 2, 3). So, we examined the co-effects of clonidine and RV on the Akt/eNOS pathway (Fig. 6). After incubation of the aortic rings with RV in the presence or absence of clonidine, the total protein levels of Akt and eNOS were not altered in diabetic mice or controls (Figs. 6A–C). However, after incubation of the aortas with clonidine in the presence of RV, the phosphorylation levels of Akt at Ser473 and eNOS at Ser1177 were both significantly increased in diabetic mice as compared with controls (Figs. 6D, E). Interestingly, only in diabetes were the levels of phosphorylated Akt significantly increased after treatment with RV in the presence rather than absence of clonidine. Moreover, RV also led to increases in phosphorylated eNOS in diabetic mice. Moreover, we studied clonidine-stimulated eNOS phosphorylation at Thr495 in the aortas from DM mice (data not shown). The RV-stimulated phosphorylation level of eNOS at Thr495 was significantly greater in aortas from DM mice than in those from controls. These phosphorylation responses were suppressed by clonidine in aortic rings from DM mice. The results demonstrated that RV activated the phosphorylation of eNOS not only at

Fig. 6. Clonidine Activates Akt/eNOS Signaling Pathway in Aortas in the Presence of RV
(A) Western blot analysis of aortas. (B) Total Akt, (C) Total eNOS, (D) Akt phosphorylation on Ser473, and (E) eNOS phosphorylation on Ser1177 expression in aortic rings. Ratios were calculated for the optical density of Akt or eNOS over that of β-actin. All samples were stimulated with RV (10^-6 mol/L) for 30 min, after some samples had been added to clonidine (10^-4 mol/L) for 20 min. Values are the mean ±S.E., n=6, **p<0.01, ***p<0.001 vs. RV+clonidine-stimulated Controls, ##p<0.01 vs. RV-stimulated DM.
Ser177 but also at Thr495.

DISCUSSION

The principal findings of this study were as follows: (1) RV markedly enhanced endothelium-dependent NO-mediated relaxations in aortic rings from control mice. Furthermore, these effects were by Akt/eNOS-dependent mechanisms. (2) RV-induced relaxation and NO production were significantly decreased in the presence of DM. We suggest that the reduction of Akt/eNOS activity played a causative role. (3) RV prevented the impairment of clonidine-induced endothelial relaxation in DM partly through augmentation of eNOS activity by an Akt-dependent mechanism (Fig. 7). We demonstrated a novel mechanism for the vasoprotective effects of the polyphenol RV in diabetic endothelial function, namely the improvement of the endothelial function in diabetes through inhibition of the Akt/eNOS pathway.

Both prospective and case-controlled clinical studies suggest a negative correlation between the consumption of polyphenol-rich foods (i.e., fruits, vegetables, cocoa) or beverages (i.e., red wine, grape juice, tea) and the incidence of cardiovascular diseases and stroke. Although the polyphenol RV has been shown to have multiple potential health benefits, including cardiovascular protection, the underlying mechanisms remain to be elucidated, it may also be due to the direct action of RV on blood vessels. Indeed, RV is able to enhance the protective effect of endothelial cells and circulating blood by increasing the endothelial formation of vasoprotective factors including NO. Such a possibility is supported by the fact that RV induces concentration-dependent relaxations of isolated blood vessels only in the presence of a functional endothelium.

As presented above, RV can relax isolated blood vessels. In the present study, we observed that L-NNA or Akt inhibitor completely abolished RV-mediated relaxations. This strongly suggests that RV causes endothelium-dependent NO-mediated relaxation via the Akt pathway. Furthermore, in this study, we report that the RV-induced relaxation response and NO production via the Akt/eNOS pathway are decreased in aortas from DM mice. The observation that RV decreased Akt and eNOS phosphorylations in aortas from DM mice (Fig. 3) suggests that RV is not able to acutely activate eNOS to increase NO production. This is the first report about the effects of single RV treatment on diabetes (Fig. 3), although this acute activation of eNOS is consistent with a previous report showing that RV induced endothelial NO production.

The effect of RV on the endothelium resembles that of clonidine, an α2-adrenergic receptor agonist. In our previous study, we reported that the clonidine-induced relaxation response in DM mice decreased through the inactivation of the Akt/eNOS signaling pathway. Furthermore, our studies performed with the diabetic mouse model (same as the diabetic mouse model which we used in this study) have also established that clonidine-stimulated diabetic aortas suppress the level of phosphorylation of eNOS in an Akt-dependent manner, resulting in the decreased production of NO. In a recent study, we observed that diabetic aortas attenuated the endothelial-dependent relaxation response induced by clonidine. The results of the present study support the effects of clonidine on endothelial function in diabetes. Therefore, we tested a hypothesis that RV enhances the impaired clonidine-induced relaxation response in DM. The clonidine-induced endothelium-dependent relaxations are markedly increased by RV in DM mice, indicating the involvement of Akt/eNOS activity. In response to clonidine, Akt and eNOS are rapidly activated in the presence of RV in aortas from DM mice (Fig. 6). Our data from Western blotting are consistent with the clonidine-induced relaxation response in the presence of RV. However, RV-induced vasodilation and NO production in the aortas from DM mice were weakened compared to those of control mice (Fig. 2), but RV-treated DM aortas augmented eNOS phosphorylation at Ser177 (Fig. 6). However, it is difficult for us to explain this discrepancy. So, we studied clonidine-stimulated eNOS phosphorylation at Thr495 in the presence or absence of RV (data not shown). eNOS has several phosphorylation sites, among which Thr495 negatively controls NO bioactivity. Although RV-stimulated DM aortas augmented the phosphorylation of eNOS at Ser177 (Fig. 6), the RV-stimulated phosphorylation level of eNOS at Thr495 was significantly greater in aortas from DM mice than in those from controls. Moreover, these phosphorylation responses were suppressed by clonidine in aortic rings from DM mice. The results demonstrated that RV activated the phosphorylation of eNOS not only at Ser177 but also at Thr495. It may follow that although RV-stimulated DM aortas augmented the phosphorylation of eNOS at Ser177, RV-induced vasodilation and NO production in those from DM mice were decreased to be counterbalanced by the effect of activated eNOS phosphorylation at Thr495. Surprisingly, RV caused an impairment of the clonidine-induced relaxation response in the controls, suggesting that the presence of RV could account for the defective endothelial vasodilation in controls. In response to clonidine, the activations of Akt and eNOS are markedly inhibited in the presence of RV in aortas from controls. We do not know why the vascular inhibitory effect happens under control conditions after RV treatment. The underlying mechanisms are unclear. RV-induced NO via Akt/eNOS activation (Fig. 1C). Possibly because Akt activation reached...
the maximum by RV pretreatment, clonidine stimulation may not have caused Akt activation in control aortas. Therefore, we concluded that the control aortas have, at least in part, a limitation regarding Akt activation. However, the molecular mechanisms by which RV increased clonidine-induced Akt/eNOS signaling in DM mice and decreased those responses in controls (Fig. 6) remains to be determined. In this study, we have shown that RV significantly increased clonidine-induced Akt and eNOS phosphorylations only in DM mice. This is the first report about the effects of RV-clonidine co-treatment on diabetes (Fig. 6). Whether the mechanisms are activated by RV or a target of RV remains to be studied in the future.

In conclusion, the present findings indicate that RV causes endothelium-dependent NO-mediated relaxation in aortic rings, which are weaker in DM mice than controls, most likely due to the decreased activation of Akt/eNOS. Furthermore, RV ameliorated the impaired clonidine-induced relaxation via Akt/eNOS activation only in DM mice. The results suggest that the additional exposure to RV and clonidine has an augmenting effect on the Akt/eNOS signaling pathway under diabetic conditions. More studies are needed, such as on the role of Akt/eNOS in the protective synergistic effect of RV and clonidine, and the possible mechanisms involved the effects of RV on controlling the endothelial function.

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**Conflict of Interest** The authors declare no conflict of interest.

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