Plant polyphenols Morin and Quercetin rescue nitric oxide production in diabetic mouse aorta through distinct pathways

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ABSTRACT

Diabetic vascular complications are associated with endothelial dysfunction. Various plant-derived polyphenols benefit cardiovascular function by protecting endothelial nitric oxide (NO) production through as yet unclear mechanisms. This study compared the effects of two structurally similar polyphenols, Morin (MO) and Quercetin (QU), on endothelial function in isolated aorta from control and streptozotocin (STZ)-induced diabetic mice. Vascular function under treatment with MO, QU, and various signaling pathway modulators was measured by isometric tension in an organ bath system, NO production by chemical assay and HPLC, and changes in protein signaling factor expression or activity by western blotting (WB). Both polyphenols acted as potent vasodilators and this effect was associated with increased phosphorylation of Akt and endothelial NO synthase (eNOS). An Akt inhibitor blocked MO- and QU-induced vasorelaxation as well as Akt phosphorylation. However, inhibitors of phosphoinositide 3-kinase (PI3K) and AMP-activated protein kinase (AMPK) suppressed only QU-induced vasorelaxation, NO production, and AMPK phosphorylation. These results suggested that plant polyphenols MO and QU both promote eNOS-mediated NO production and vasodilation in diabetic aorta, MO via Akt pathway activation and QU via PI3K/Akt and AMPK pathway activation. Elucidation of these pathways may define effective therapeutic targets for diabetic vascular dysfunction.

1. Introduction

Diabetes mellitus (DM) affects a rapidly growing portion of the global population and is a strong risk factor for cardiovascular complications [1]. These DM-associated vascular complications are due in part to endothelial dysfunction stemming from chronically elevated blood glucose [2–4]. Therefore, improving endothelial function is a crucial aspect of diabetic treatment.

The endothelium is one of the largest organs in the body, and controls local vascular tone to provide sufficient perfusion pressure to target organs. Endothelial dysfunction is a major pathogenic factor in cardiovascular disease resulting from DM, obesity, hypertension, hyperlipidemia, and poor dietary habits [5]. Endothelial dysfunction accelerates the inactivation and decreases the bioavailability of nitric oxide (NO), the main diffusible factor inducing vascular smooth muscle relaxation and concomitant vasodilation [6]. Vascular NO is mainly produced by endothelial NO synthase (eNOS) [7], so therapeutic approaches targeting eNOS activation are potentially effective methods to ameliorate vascular endothelial cell impairments due to DM.

Flavonoids are a large group of polyphenolic phytochemicals [8] with a wide range of pharmacological activities, including antibacterial, anti-inflammatory, antioxidant, and antitumor effects [9,10]. Administration of polyphenolic flavonoid compounds to DM model animals has been shown to improve endothelial dysfunction and ensuing symptoms [11,12]. Quercetin (QU) is one of the major plant flavonoids commonly consumed from vegetables and fruits in the human diet and is widely believed to benefit health through potent vasodilator, free radical-scavenging, and antioxidant actions [13,14]. In addition, QU has been shown to improve endothelial–dependent vasodilation by protecting against reduced NO production and bioavailability in both in vitro and in vivo studies of diabetic rats [11,14,15]. Morin (MO) is a plant polyphenol with a structure similar to QU (Fig. 1), differing only in the position of a single hydroxyl group (Fig. 1). Morin was originally isolated from members of the Moraceae family and subsequently extracted from the leaves, fruits, stems, and branches of numerous plants [16,17]. Recently, we reported that MO ameliorates endothelial dysfunction in DM mice via Akt/eNOS signal activation [16]. However, little is known of the upstream mechanisms by which MO benefits endothelial dysfunction in DM. Both QU and MO have been suggested as dietary factors that can improve vascular function in DM [16,18]. New insight into the physiological actions of QU and MO and the underlying molecular mechanisms may identify novel pathogenic processes and...
cleaned of excess connective tissue and fat, and cut into rings 2 mm in
length. Aortic rings were attached to a force transducer (model TB-
611T; Nihon Kohden, Tokyo, Japan) in an organ bath system contain-
ing KHS maintained at 37°C and continuously aerated with a
mixture of 5% CO2 and 95% O2. In all experiments, special care was
taken to avoid damaging the luminal surface of the endothelium. The
rings were equilibrated for 45 min at a resting tension of 1.5 g before
the experiment. During the equilibration period, the rings were washed
every 15 min. After equilibration, all segments were stimulated with
potassium chloride (80 mM) to determine the maximum contractility of
smooth muscle. Rings were preconstricted with a submaximal concen-
tration of prostaglandin F2α (PGF2α; 10⁻⁶ to 3 × 10⁻⁶ M) (Fuji
pharma, Tokyo, Japan) producing a force of approximately 1.0 g to
evaluate vascular relaxation responses to acetylcholine (ACh; 10⁻⁹ to
10⁻⁵ M) (Daichi-Sankyo Pharmaceuticals, Tokyo, Japan), sodium ni-
troprusside (SNP; 10⁻¹⁰ to 10⁻⁵ M) (Wako Chemical), MO (10⁻⁹ to 10⁻⁵
M) (Sigma Chemical, St. Louis, MO, USA), and QU (10⁻⁹ to 10⁻⁵ M)
(Sigma Chemical), and with/without other signaling modulators as
indicated. To examine the effects of MO or QU on responses to ACh and
SNP, rings were preincubated for 30 min with MO or QU (10⁻⁶ M,
respectively) before ACh and SNP. To assess the contribution of NO to
MO and QU responses, rings were preincubated for 30 min with the
NOS inhibitor N⁶-nitro-L-arginine (L-NNA; 10⁻⁴ M) (Sigma-Aldrich, St.
Louis, MO, USA). To determine the mechanisms underlying responses
to MO and QU, rings were incubated with the Akt inhibitor 1-L-6-hy-
droxymethyl-chiro-inositol 2(2R)-2-0-methyl-3-O-octadecyl-sn-glycer-
carbonate) (10⁻⁶ M) (Calbiochem, San Diego, CA, USA), the phos-
phoinositide 3-kinase (PI3K) inhibitor LY294002 (LY; 10⁻⁶ M)
(Calbiochem), or the AMPK inhibitor Compound C (CC; 5 × 10⁻⁶ M)
as indicated for 30 min before MO or QU application. The preconstric-
tion induced by PGF2α was normalized to the aortic response by adjusting the dose of PGF2α(10⁻⁶ to 3 × 10⁻⁶ M). Thus, the preconstric-
tion had been fixed in the presence or absence of either MO or QU.

2.3. Measurement of aortic nitrate/nitrite level
Total NO production from the aorta was measured using an auto-
mated NO detector/high-performance liquid chromatography system
(ENO20; Eicom, Kyoto, Japan) according to the manufacturer’s pro-
tocol as described previously [16,19,20]. Isolated aorta was cleared of
surrounding tissue, cut into 4-mm sections, and preincubated in KHS at
37°C. Sections were then preincubated in the presence of Akt inhibitor
(10⁻⁶ M), LY (10⁻⁶ M) or CC (10⁻⁶ M) as indicated for 30 min prior to
addition of MO or QU (10⁻⁶ M) for 20 min. Finally, aorta sections were
semi-dried, weighed, and frozen in liquid nitrogen. The frozen aortas
were lyzed for western blotting (described below) to measure changes
in expression or phosphorylation of various signaling factors. NO pro-
duction (NOx; nitrate + nitrite level) is expressed in mol/g weight of
the aorta /min.

Fig. 1. Chemical structures of the plant polyphenols Quercetin (QU) and Morin (MO). Structures differ only by the position of a single hydroxyl group.
2.4. Western blotting

Whole frozen aorta was lysed immediately after treatment in RIPA buffer (Thermo Scientific, Rockford, IL, USA), and total protein quantified using the Pierce BCA protein assay kit (Thermo Scientific). Equal amounts of total protein per gel lane were separated and blotted for estimation of total Akt (1:1000; #9272 from Cell Signaling Technology, Danvers, MA, USA) and phospho-Akt Ser473 (1:1000; #9271 from Cell Signaling Technology), total eNOS (1:1000; 610,296 from BD Bioscience, NJ, USA) and phospho-eNOS Ser1177 (1:1000; #9571 from Cell Signaling Technology), AMPK (1:1000; #2532 from Cell Signaling Technology), and phospho-AMPK Thr172 (1:1000; #2535 from Cell Signaling Technology), PI3K (p85; 1:1000; #4292, p110α; 1:1000; #4255 from Cell Signaling Technology), and β-actin as a gel loading control (1:5000; A5316 from Sigma Chemical, St. Louis, MO, USA). Protein signaling activity was quantified as the level of phospho-protein divided by total protein after normalization to β-actin expression.

2.5. Statistical analysis

Relaxation responses to ACh, MO, QU and SNP were calculated as the percentage of force from maximum PGF$_{2α}$ contraction. Data are presented as mean ± standard error (SE) for n animals. Student’s t-test was used to assess differences between two mean values. Multiple dose-response curves were compared using repeated-measures 2-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. NO production and protein expression were compared among groups by one-way ANOVA. A $p < 0.05$ was considered significant for all tests. All analyses were conducted using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. The characteristics of STZ-induced DM mice

STZ-induced DM mice (at 12–16 weeks post-treatment) displayed body weight loss and a non-fasting plasma glucose value over 400 mg/dL (Table 1), which was consistent with the results of previous study [16]. Based on these observations, we judged that the STZ-induced DM model we used in this study indeed caused type 1 DM.

3.2. Effects of acute MO and QU treatment on aortic vasoreactivity in DM mice

ACh (10$^{-9}$ to 10$^{-5}$ M) induced dose-dependent aortic relaxation following precontraction by PGF$_{2α}$ in all treatment groups (Fig. 2A and B). In aortic rings from vehicle-injected Control mice, this ACh-induced relaxation was not significantly changed by MO or QU pretreatment (Fig. 2A). On the other hand, DM induction blunted ACh-induced relaxation (Fig. 2B), and pretreatment with MO or QU (30 min) substantially enhanced ACh-induced (endothelial-dependent) relaxation compared to corresponding vehicle controls (Fig. 2B). As expected, the NO donor SNP-induced vasorelaxation in all treatment groups (Fig. 2C and D) and responses did not differ between Control and DM mice either in the presence or absence of MO or QU pretreatment.

3.3. Role of NO in MO- and QU-induced aortic relaxation

Treatment with MO alone (10$^{-9}$ to 10$^{-5}$ M) also induced dose-dependent vasorelaxation in Control aorta (Fig. 3A), a response that was significantly enhanced in aortic rings from DM mice (Fig. 3B). QU alone
(10^-9 to 10^-5 M) also induced relaxation (Fig. 3A) but the responses to MO alone were augmented relative to responses in DM aortas (Fig. 3B). In aortas from both Control and DM mice, the relaxant responses induced by MO and QU were abolished by pretreatment with the NOS inhibitor L-NNA (10^-4 M) for 30 min. B, D; Relaxation responses of DM aortas to MO or QU in the absence (B) or presence (D) of L-NNA for 30 min. Values expressed as mean ± SE. n = 8 rings from eight mice. ###p < 0.001 vs. QU. 

Fig. 3. MO- and QU-induced relaxation responses (A-D) and associated NO production (E) in aortas from DM and Control mice. A, C; Relaxation responses of Control aortas to MO or QU in the absence (A) or presence (C) of the NOS inhibitor l-NNA (10^-4 M) for 30 min. B, D; Relaxation responses of DM aortas to MO or QU in the absence (B) or presence (D) of l-NNA for 30 min. Values expressed as mean ± SE. n = 8 rings from eight mice. ###p < 0.001 vs. QU. E; Influence of ACh, MO, and QU on NO production in Control and DM aortas. Aortas were incubated with 10^-6 M ACh, MO, or QU for 20 min. NO levels were measured in the KHS. Values expressed as mean ± SE. n = 6 rings from six mice. *p < 0.05 vs. Control under ACh stimulation. ###p < 0.001 vs. DM under ACh stimulation. &p < 0.05 vs. Control under MO stimulation. !p < 0.05 vs. DM under MO stimulation.

3.4. Contributions of Akt, PI3K, and AMPK signaling to QU-induced aortic relaxation

Next, we examined the contributions of PI3K, Akt, and AMPK signaling pathways to modulation of aortic relaxation by QU. These QU-induced vascular relaxations were significantly attenuated by an Akt inhibitor or PI3K inhibitor (LY294002) in aortas from both Control and DM mice (Fig. 4A and B), indicating that PI3K/Akt signaling is required for QU-induced aortic relaxation. In the presence of Compound C, QU-induced aortic relaxation was significantly inhibited in only DM aorta (Fig. 4B) while there was no change in Control aorta (Fig. 4A).

To further confirm the involvement of Akt, PI3K, and AMPK, rings were incubated with QU in the presence of specific inhibitors (Akt inh, LY294002, and Compound C, respectively) and the levels of NO measured in the KHS (Fig. 4C). QU-induced production of NO was significantly reduced by Akt inh or LY294002 compared to non-stimulation in both Control and DM aortas, while Compound C pretreatment significantly inhibited QU-induced NO production only in DM aortas (Fig. 4C). These findings suggest that QU increases NO levels via PI3K/Akt signaling in Control aorta but by PI3K/Akt and AMPK signaling in
We previously demonstrated that MO-induced vascular relaxation was significantly reduced by an Akt inhibitor and by L-NNA [16]. Here we examined if MO modulates NO production via PI3K/Akt and (or) AMPK, as AMPK is also known to regulate NO production by phosphorylating eNOS at Ser1177 [21,22]. We confirmed that preincubation with Akt inhibitor suppressed MO-induced vasorelaxation in aorta from both Control and DM mice (Fig. 5A and B). However, there was no significant difference in MO-induced vasorelaxation in the presence of the PI3K inhibitor LY294002 in either Control or DM aorta (Fig. 5A and B). Moreover, Compound C did not suppress MO-induced vasorelaxation in either Control or DM aorta (Fig. 5A and B).

To further examine the participation of Akt, PI3K, and AMPK signaling in MO-induced vasorelaxation, we measured NO production from MO-treated rings preincubated with Akt inhibitor, LY294002, or Compound C. MO-induced NO production was significantly inhibited by the Akt inhibitor in both Control and DM aortas, but the magnitude of the decrease was greater in DM aorta (Fig. 5C). Alternatively, the MO-induced increase in NO production was not markedly altered by LY294002 or Compound C (in fact, NO production was slightly elevated in both Control and DM aorta). Collectively, these findings implicate activation of the Akt signaling axis in MO-induced aortic relaxation, while PI3K and AMPK signaling pathways do not appear to contribute.

**3.6. Effects of MO and QU on expression levels of Akt, eNOS, AMPK, and PI3K in Control and DM aortas**

To examine if MO- and QU-induced NO production is associated with changes in the expression or phosphorylation (activity) levels of Akt, eNOS, and PI3K, we conducted western blotting of aortic lysates following the indicated treatments (Fig. 6). Expression of p-Akt increased dramatically in both MO-stimulated Control and DM aorta and in QU-stimulated Control aorta compared to unstimulated Control and DM aortas (Fig. 6A and B). MO also significantly increased p-eNOS expression in both Control and DM aortas compared to corresponding unstimulated aortas, while QU significantly increased p-eNOS expression only in DM aorta (Fig. 6A and C). Further, QU upregulated p-AMPK expression in DM aorta but not Control aorta, while MO had no effect on either group (Fig. 6A and D). Neither MO nor QU altered PI3K p85 and 110α subunit protein expression levels in Control and DM aortas (Fig. 6A, E, and F). These results suggest that both MO and QU may activate Akt/eNOS signaling, while QU may also activate AMPK/eNOS signaling in DM aorta.

To further investigate the mechanisms of QU-induced eNOS activation, we measured the effects of the aforementioned specific inhibitors (Fig. 7A and B). Expression of p-Akt was significantly elevated by QU in Control aorta, and both the Akt inhibitor and LY294002 dramatically decreased p-Akt expression levels. These results suggest that QU upregulates p-Akt expression via activation of PI3K pathway. QU also enhanced expression of p-Akt in DM aorta (Fig. 7C and D), a response suppressed by the Akt inhibitor or LY294002 treatment. However, Compound C treatment did not alter this QU-stimulated increase in p-Akt expression. This result suggests that Akt may act downstream of PI3K rather than AMPK in QU-stimulated DM aorta.
Expression of p-eNOS was also dramatically increased by QU in DM aorta (Fig. 7C and E). Treatment with Akt inhibitor, LY294002, or Compound C significantly decreased p-eNOS expression in QU-stimulated DM aorta suggesting that QU upregulated p-eNOS via PI3K/Akt and AMPK pathway. QU also increased p-AMPK expression in DM aorta (Fig. 7C and F), an effect abolished by Compound C, but not the Akt inhibitor or LY294002, suggesting that QU-induced AMPK activation is independent of QU-induced PI3K/Akt signaling in DM aorta.

4. Discussion

In the present study, we demonstrate that MO and QU protect against endothelial dysfunction in STZ-induced DM aorta, but via distinct signaling pathways. MO strongly increases eNOS activation, NO production, and ensuing vascular relaxation via Akt signaling independent of PI3K and AMPK, while QU increases eNOS activation, NO production, and vascular relaxation in DM aorta via PI3K/Akt and AMPK signaling (Fig. 8).

There are at least two major pathways to mediate the activation of eNOS in endothelial cells. Activation of the PI3K/Akt signaling pathway leads to the phosphorylation of eNOS at Ser1177 and subsequent NO production. Meanwhile, ACh induces a rapid increase in intracellular Ca\(^{2+}\) in endothelial cells activating the Ca\(^{2+}\)-dependent calmodulin/eNOS signaling pathway, resulting in the release of NO. ACh-induced Ca\(^{2+}\)/eNOS signal activation pathway is a classical endothelial function. Where we evaluated the relationship between the classical function, and MO and QU, we know that MO induces vascular relaxation via activation of Akt/eNOS signaling [16]. So, we evaluated the MO and QU and ACh in this study. ACh-induced vasorelaxation is endothelium-dependent and mediated by NO [23]. This response was markedly reduced in aortas from DM mice, in accord with previous reports [16,18,20]. Impaired ACh-induced vasorelaxation suggests attenuation of endothelial NO release as a potential pathogenic mechanism in DM. Notably, both MO and QU pretreatment reversed blunted ACh-induced vasorelaxation and enhanced NO production. Further, the effects of both agents were endothelial dependent as SNP, a general NO donor and endothelial-independent vasodilator, had no effect on MO- or QU-induced vasorelaxation in DM aorta. Moreover, responses to both agents were suppressed by the NOS inhibitor L-NNA. SNP-induced vasorelaxation also did not differ between Control and DM groups, consistent with the majority of previous studies [16,24] suggesting that NO production or bioavailability rather than vascular smooth muscle function is impaired in DM. Collectively, these findings suggest that DM-associated vascular dysfunction may be improved by augmenting the NO pathway in endothelial cells. In addition, these results are consistent with previous reports the flavonoids exert beneficial vascular effects primarily by enhancing endothelial-derived NO production [25].

As previously described [16,26], both MO and QU induced vasorelaxation in Control and DM aorta, and we confirmed that these responses were endothelial-dependent. Notably, MO was a more potent inducer of vasorelaxation and NO production than QU in DM mice, while both agents were equally effective in modulating ACh-induced relaxation. However, our results do not provide conclusive evidence for the superiority of MO over QU for augmenting NO production or vasorelaxation. Rather, it is reasonable to speculate that both MO and QU may be effective against endothelial dysfunction in DM. The in vivo efficacy of both compounds warrants further investigation.

The enhanced NO production observed under both MO and QU treatment suggests enhanced eNOS activity. Activation of eNOS is dependent on an increase in intracellular calcium concentration, as occurs when ACh activates endothelial muscarinic receptors [27]. However, eNOS can also be activated by resting calcium when phosphorylated on Ser1177 by Akt, possibly also releasing NO in DM aorta.
specific amino acids residues. Some agonists induce vasorelaxation by activating the Akt signaling pathway, with subsequent phosphorylation of eNOS on Ser1177 [19,20,28]. Indeed, both MO and QU increased eNOS Ser1177 phosphorylation in DM aorta (although only MO enhanced p-eNOS in controls).

Similarly, both MO and QU-induced vascular relaxation by
activating Akt as the effects were blocked by an Akt inhibitor. The PI3K/Akt pathway is a critical regulator of normal endothelial function [29]. Many studies have investigated vascular endothelial-dependent relaxation responses via the PI3K/Akt/eNOS signaling pathway [28,30–33]. PI3K/Akt pathway activation can promote eNOS expression and elevate NO production [34,35]. Furthermore, activation of vascular endothelial AMPK has been shown to phosphorylate eNOS on Ser1177, stimulating NO release and subsequent relaxation of large vessels [21,22]. Both PI3K and AMPK in addition to Akt contributed to QU-induced NO production and vasorelaxation in DM aortas, while vasorelaxation induced by MO was altered only by Akt blockade. Thus, the effect of MO could be direct Akt activation, although this requires additional study.

Akt, PI3K, and AMPK are expressed in vascular endothelial cells [29,36,37]. In previous studies, polyphenol-induced endothelial formation of NO was mediated by eNOS phosphorylation via the PI3K/Akt pathway [38,39]. NO, an endothelial-derived vasodilator, plays a chief role in the regulation of endothelial function homeostasis [16,18–20]. We used L-NNA, an eNOS inhibitor, to block the synthesis of NO by endothelial synthases. Pretreatment with L-NNA caused a substantial
attenuation of MO- and QU-induced relaxation responses. Thus, this study also demonstrates that the endothelial AMPK/PI3K/Akt/eNOS pathway accounts for adequate endothelial functioning in whole aorta. In the present study, vasorelaxation induced by QU was accompanied by increased p-Akt, p-eNOS, and NO production. AMPK is an important sensor of cellular energy status, including in endothelial cells [36,37], and previous studies have shown that AMPK also induces eNOS phosphorylation at Ser1177, thereby enhancing NO generation in endothelial cells [21,22]. Moreover, AMPK is activated by dietary flavonoids [40,41], and others have demonstrated that AMPK activation can prevent vascular dysfunction via increased phosphorylation and activation of eNOS [42,43]. Shen et al. [41] in their study showed that QU-induced the activation of AMPK/eNOS signal pathway under serum-starved conditions in human aortic endothelial cells. Therefore, AMPK may be an important link between cellular energy status and endothelial function. In our present study, QU enhanced AMPK phosphorylation only in aorta from DM mice as confirmed by pharmacological blockade. Inhibition of AMPK also prevented QU-mediated phosphorylation of eNOS but not Akt phosphorylation in DM aorta. Inhibition of AMPK also partially blocked QU-induced vasorelaxation and NO production, but only in DM aorta. Therefore, our data suggest the acute protective effect of QU is mediated in part via an AMPK/eNOS-dependent pathway as well as the PI3K/Akt/eNOS pathway in DM. However, whether QU and MO have similar effects on human aortic endothelial cells needs to be evaluated in subsequent studies that are beyond the scope of the present study.

5. Conclusion

While these two flavonoids activate distinct signaling pathways, their structures are very similar. Our findings suggest that MO may exert potential therapeutic effects for the prevention and treatment of endothelial dysfunction in DM through phosphorylation of Akt and eNOS, while QU is also able to induce eNOS activation and increase NO production in DM via PI3K and AMPK. In summary, the present study provides further evidence for the protective efficacy of plant-derived polyphenols against diabetic vascular complications.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Fig. 8. The potential mechanisms of ACh-, MO-, and QU-induced eNOS activation and vascular relaxation. In Control, MO activates eNOS via the Akt signaling pathway independent of PI3K and AMPK, while QU activates eNOS via the PI3K/Akt signaling pathway, independent of AMPK. ACh/eNOS/NO is the major pathway under control conditions. In DM, vascular endothelial dysfunction involves reduced eNOS activity. MO activates eNOS via the Akt signaling pathway, while QU activates eNOS via the PI3K/Akt and AMPK signaling pathways.
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