Original Paper

Cinnamaldehyde Prevents Endothelial Dysfunction Induced by High Glucose by Activating Nrf2

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Key Words

Cinnamaldehyde • Diabetes • NF-E2-related factor 2 • Endothelium • Oxidative stress

Abstract

Background/Aims: It is well documented that hyperglycemia-induced oxidative stress is an important causative factor of endothelial dysfunction. Cinnamaldehyde (CA) is a key flavor compound in cinnamon essential oil that can enhance the antioxidant defense against reactive oxygen species (ROS) by activating NF-E2-related factor 2 (Nrf2), which has been shown to have a cardiovascular protective effect, but its role in endothelial dysfunction induced by high glucose is unknown. **Methods:** Dissected male C57BL/6J mouse aortic rings and HUVECs were cultured in normal glucose (NG 5.5 mM) or high glucose (HG 30.0 mM) DMEM treatment with or without CA (10 μM). **Results:** Treatment with CA protected the endothelium relaxation, inhibited ROS generation and preserved nitric oxide (NO) levels in the endothelium of mouse aortas treated with high glucose. CA up-regulated Nrf2 expression, promoted its translocation to the nucleus, and increased HO-1, NQO1, Catalase and Gpx1 expression under high glucose condition. The increased level of nitrotyrosine in HUVECs under high glucose was also attenuated by treatment with CA. Dihydroethidium (DHE) and DAF-2DA staining indicated that CA inhibited the ROS generation and preserved the NO levels in HUVECs, but these effects were reversed by Nrf2-siRNA in high glucose conditions. **Conclusion:** Our results indicated that CA protected endothelial dysfunction under high glucose conditions and this effect was mediated by Nrf2 activation and the up-regulation of downstream target proteins. CA administration may represent a promising intervention in diabetic patients who are at risk for vascular complications.

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Introduction

Diabetes mellitus (DM) is a major and an increasing health problem worldwide. Its related vascular complications are the major cause of morbidity and mortality [1]. Studies have shown that markers of chronic, low-grade inflammation are correlated with markers of endothelial dysfunction in diabetic patients without clinical signs of macrovascular disease, such as ischemic heart disease and stroke [2]. It is well documented that the major cause of this inflammatory injury can be attributed to hyperglycemia-induced reactive oxygen species (ROS) generation [3]. Given the importance of oxidative stress in diabetic vascular complications, anti-oxidative stress treatments are thought to be an important intervention with the potential to ameliorate hyperglycemia-induced vascular lesions. However, the use of exogenous radical scavengers, such as vitamin C and vitamin E, in large clinical trials remains controversial [4, 5].

The endogenous antioxidative enzyme NF-E2-related factor 2 (Nrf2) is thought to function as a physiological regulator of ROS generation and may contribute to the prevention of diabetes-related cardiovascular diseases [6]. Nrf2 regulates the expression of numerous genes, such as heme oxygenase 1 (HO-1) and NAD(P)H dehydrogenase, quinone 1 (NQO1), by interacting with antioxidant response elements in target gene promoters, and the protein products of these genes neutralize free radicals and accelerate the removal of environmental toxins [6].

Previous studies indicated that cinnamic aldehyde (CA), the key flavor compound in cinnamon essential oil extracted from *Cinnamomum zeylanicum* and *Cinnamomum cassia* bark, could reduce disease onset or improve prognosis [7]. CA enhanced the antioxidant defense against ROS produced under hyperglycemic conditions and thus protected pancreatic beta cells and exhibited anti-diabetic properties [8]; it also prevented the development of hypertension in insulin deficiency and insulin resistance by normalizing vascular contractility and exerting an insulintropic effect [9]. Additionally, CA induces endothelium-dependent vasorelaxant activity in isolated rat aortas [10]. More importantly, CA is a potent Nrf2 inducer, and when used at low doses incapable of eliciting cytotoxicity, it may thus serve as a cancer chemopreventive agent [11]. CA activates the Nrf2-dependent antioxidant response in human epithelial colon cells [12]. In a streptozotocin-induced diabetes model, CA has an Nrf2-dependent effect on the antioxidant status of the rat kidney [7]. The above studies indicated that CA has beneficial effects for diabetes and related complications. However, the direct effects and underlying mechanism of CA on endothelium dysfunction induced by high glucose are unknown.

The present study was performed to test the hypothesis that CA attenuates high glucose-induced endothelial dysfunction through an Nrf2-mediated antioxidant effect.

Materials and Methods

Artery and endothelial cell culture

Eight week old male C57BL/6J mouse (Model Animal Research Center, Nanjing University, Jiangsu, PR China) aortic rings were dissected in sterile PBS and incubated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 IU/mL penicillin and 100 µg/mL streptomycin. The high-glucose (HG: 30 mM) condition was achieved by the addition of 24.5 mM D-glucose, and 24.5 mM of mannitol was used in the normal glucose (NG) osmotic control condition [13]. After the 24-h incubation period, the aortic rings were transferred to a chamber filled with fresh Krebs solution and mounted in a wire myograph (DMT 620_M, Denmark) to measure changes in the isometric force. HUVECs (CHI Scientific, Inc, China) were grown in DMEM supplemented with 10% FBS and 1% antibiotics. Cultured aortic rings and cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO₂. The cells were made quiescent by the incubation of 90% confluent cultures in serum-free DMEM and were then incubated with CA (10 µM) for 24 h in the presence or absence of Nrf2 siRNA.

Cinnamaldehyde treatment

CA (Sigma-Aldrich, USA) diluted in DMSO was added to the incubation medium at the same time when the cells or artery treated by high glucose or normal glucose. The control group was treated by DMSO (final concentration was less than 0.1 %).

RNA interference of Nrf2

Small interfering RNAs (siRNAs) specific for Nrf2 were purchased from Santa Cruz Biotechnology, Inc. (USA), and the transfection of siRNAs was performed according to the manufacturer's instructions.

Measurement of vascular activities

Changes in the isometric tone of the aortic rings were recorded by wire myograph, as previously described [13]. The arterial segments were stretched to an optimal baseline tension (2 mN) and then allowed to equilibrate for one hour before being contracted with 60 mM KCl and rinsed in Krebs solution. Endothelium-dependent relaxation was measured by testing the concentration-response relationship upon the cumulative addition of acetylcholine (ACh) to phenylephrine (Phe)-precontracted rings. The endothelium-independent relaxation response to nitroglycerine (NTG) was also measured in artery rings.

Measurement of ROS production

To assess superoxide production, dihydroethidium (DHE; Sigma-Aldrich, USA) staining was performed according to a previously described method. Aortic segments and cells were incubated with 40 μ M DHE for 45 min at 37°C, after which the sections or cells were washed three times in DHE-free Krebs solution, the aortic sections were cut open, and the endothelium was inverted and placed between two coverslips for microscopic analysis [14]. Images were acquired, and the fluorescence intensity was analyzed.

Evaluation of NO levels

The NO levels in HUVECs and aortic segments were assessed by staining with diaminofluorescein-2 diacetate (DAF-2 DA, Sigma-Aldrich, USA) in Krebs solution for 45 min at 37°C followed by three washes with Krebs solution. Arteries were prepared as described above. The NO fluorescence was detected and the fluorescence intensity was analyzed as described above.

Western blotting analysis

Immunoblots of Nrf2, nitrotyrosine, NQO1, HO-1, Catalase (CAT), Glutathione peroxidase 1 (GPx-1) and GAPDH were prepared as previously described [15]. After incubation with secondary antibodies (ZSGB-BIO, China) at room temperature for 2 h, the proteins were detected with enhanced chemiluminescence and quantified using a Gel Doc 2000 Imager (Bio-Rad, USA). Protein expression was normalized to GAPDH. All of the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

Data are the means $\pm$ SEM. The maximum response (E_{max}) was calculated from individual agonist concentration-response curves using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). The significant differences in mean values were assessed by Student's *t*-test. Two-sided *P* values <0.05 were considered statistically significant.

Results

Cinnamaldehyde preserved in vitro endothelium-dependent relaxation of mice aortas under high glucose

High-glucose (HG) exposure for 24 h impaired the ACh-induced endothelium-dependent relaxation of aortic rings compared with the normal-glucose (NG) exposure condition. The presence of CA (10 μ M) in high glucose preserved the endothelium-dependent relaxation (Fig. 1A). In normal glucose condition, CA improved ACh induced relaxation slightly, but there were no significant differences between the CA group and Control group, $P > 0.05$ (Fig. 1A). The endothelium-independent relaxation induced by nitroglycerin was similar in each group (Fig. 1B).

Fig. 1. Cinnamaldehyde preserved *in vitro* endothelium-dependent relaxation of mice aortas under high glucose conditions. (A) High-glucose exposure (HG, glucose 30 mM, 24 h) impaired the endothelium-dependent relaxation of mouse aortas *in vitro*. Cinnamaldehyde (CA, 10 μ M) treatment ameliorated endothelial dysfunction in mouse aortas under high-glucose conditions but not in normal-glucose conditions. $^{**}P < 0.01$ versus the NG group; $^{##}P < 0.01$ versus the HG group. (B) Endothelium-independent relaxation is shown for all groups tested. Data are the means $\pm$ SEM. In each case, $n = 6$ samples from different mice.

Fig. 2. Cinnamaldehyde inhibited ROS production and preserved NO levels in mice aortas treated with high glucose *ex vivo*. (A and C) ROS production measured by DHE staining in the *en face* endothelium of aortas from C57BL/6J mice after treatment with NG (NG group), NG plus CA (NG+CA, 10 μ M), HG (HG group) or HG plus CA (HG+CA, 10 μ M). (B and D) NO production measured by DAF-2DA staining in the *en face* endothelium of aorta arteries. Red: DHE fluorescence. Green: DAF-2DA fluorescence. $^{**}P < 0.01$ versus the NG group; $^{##}P < 0.01$ versus the HG group. Data are the means $\pm$ SEM. In each case, $n = 6$ samples from different mice. The scale bar indicates 20 μ m.

Fig. 2. Cinnamaldehyde inhibited ROS production and preserved NO levels in mice aortas treated with high glucose *ex vivo*. (A and C) ROS production measured by DHE staining in the *en face* endothelium of aortas from C57BL/6J mice after treatment with NG (NG group), NG plus CA (NG+CA, 10 μ M), HG (HG group) or HG plus CA (HG+CA, 10 μ M). (B and D) NO production measured by DAF-2DA staining in the *en face* endothelium of aorta arteries. Red: DHE fluorescence. Green: DAF-2DA fluorescence. $^{**}P < 0.01$ versus the NG group; $^{##}P < 0.01$ versus the HG group. Data are the means $\pm$ SEM. In each case, $n = 6$ samples from different mice. The scale bar indicates 20 μ m.

Fig. 3. Cinnamaldehyde acts as an Nrf2 activator in high glucose-treated HUVECs. (A and B) Nrf2 expression (A) and localization (B) were assessed in HUVECs incubated in NG, HG, or HG+CA DMEM media for 24 h. Incubation in HG as well as treatment with HG+CA increased Nrf2 staining intensity and translocation to the nucleus. $**P < 0.01$ versus the NG group; $##P < 0.01$ versus the HG group. Data are the means $\pm$ SEM. In each case, $n = 3$. The scale bar indicates 10 μ m.

Cinnamaldehyde inhibited ROS production and prevented NO reduction in mouse aortas treated with high glucose in vitro

ROS production measured by DHE fluorescence in the *en face* endothelium of the aorta was higher in the HG than in the NG group. CA (10 μ M) treatment reduced ROS production in the endothelium of the aorta (Fig. 2). DAF-2DA staining indicated increased ROS levels in the *en face* endothelium of the aorta accompanied by decreased NO levels, but treatment with CA (10 μ M) preserved NO levels in the endothelium under high glucose conditions (Fig. 2A and 2B). Under normal glucose condition, CA treatment decreased the ROS levels and increased NO levels slightly, but there were no significant difference between the NG+CA group and NG group, $P > 0.05$.

Cinnamaldehyde acts as an Nrf2 activator in high glucose-treated ECs

Previous studies demonstrated that CA exerted anti-oxidative properties by activating Nrf2 in human skin and renal mesangial cells [7, 12], but whether CA has a similar effect on ECs under high glucose conditions was unknown. Western blotting indicated that the expression of Nrf2 was slightly increased under hyperglycemic conditions, and CA treatment further increased the Nrf2 levels. Immunofluorescent staining revealed predominant nuclear localization of Nrf2 in response to CA treatment. The above results indicated the activation of the Nrf2 pathway by CA treatment in ECs exposed to high glucose. We then investigated whether the induction of the Nrf2 pathway by CA could also affect the Nrf2 downstream genes, such as HO-1, NQO1, CAT and GPx-1. As shown in Fig. 4A-D, HO-1, NQO1, CAT and GPx-1 expression under high glucose condition were increased by CA. The interaction between O_2 and nitric oxide (NO) forms reactive peroxynitrite (ONOO-) [16]. We then asked whether the activation of Nrf2 could prevent tyrosine nitration in ECs treated with high glucose. Immunoblotting showed that the level of nitrotyrosine in the CA treatment group was lower than in the HG group (Fig. 4E).

Cinnamaldehyde diminished ROS levels and preserved NO levels in ECs under high glucose conditions

To determine the relationship between CA and the Nrf2 signal pathway, Nrf2 siRNA was used. We first confirmed that Nrf2 siRNA significantly blocked Nrf2 expression in high glucose-treated ECs, but control siRNA did not (Fig. 5A). We next investigated whether

Fig. 4. Effects of Cinnamaldehyde on protein expression of HO-1, NQO1, CAT, GPx-1 and levels of nitrotyrosine. (A, B, C and D) Effects of NG, high-glucose (HG, glucose 30 mM) and HG plus CA on HO-1, NQO1, CAT and GPx-1 expression levels detected by Western blotting. $**P < 0.01$ versus the normal glucose group (NG, glucose 5.5 mM); $##P < 0.01$ versus the HG group. (E) Effect of CA treatment on the nitrotyrosine levels in HUVECs observed by Western blotting. $**P < 0.01$ versus the NG group; $##P < 0.01$ versus HG group. Data are the means $\pm$ SEM. In each case, $n = 3$.

siRNA of Nrf2 could affect the downstream targets, western blotting results indicated the increased levels of HO-1 and NQO1 by CA were all reversed by siRNA of Nrf2 under high glucose condition (Fig. 5B and C). High glucose increased the ROS levels compared with NG (5.5 mM). Treatment with CA (10 μ M) diminished the ROS levels, and this effect was antagonized by Nrf2 signal interference (Fig. 5D and F). We then asked whether this effect would preserve the NO levels under hyperglycemic conditions. DAF-2DA staining indicated that CA treatment prevented the decrease of NO levels under high glucose conditions, and this effect was also blocked by Nrf2 inhibition (Fig. 5E and G). These results indicated that the CA-mediated diminution of ROS production and preservation of NO levels in ECs under high glucose is associated with Nrf2 signaling.

Discussion

In the present study, we have shown for the first time that treatment with CA prevented high glucose-induced ROS generation and preserved NO levels in the endothelium, thus preserving the endothelium-dependent relaxation but not the endothelium-independent relaxation. Furthermore, CA up-regulated Nrf2 expression and promoted its nuclear

Fig. 5. CA attenuates high glucose-induced ROS production and preserves NO levels in HUVECs via Nrf2. (A) Effects of Nrf2 siRNA on Nrf2 expression detected by Western blotting in high glucose-treated HUVECs. (B and C) Effects of Nrf2 siRNA on HO-1, NQO1 expression in high glucose plus CA (10 μ M)-treated HUVECs. (D and F) Representative images and data from DHE-stained HUVECs. High-glucose exposure increased the levels of superoxide anions in HUVECs, and CA (10 μ M) administration decreased the superoxide levels under high-glucose conditions. These effects were blocked by Nrf2 siRNA. (E and G) Representative images and data from DF-2DA-stained HUVECs. High-glucose exposure decreased the level of NO in HUVECs, and CA increased the NO level under high-glucose conditions. These effects were blocked by Nrf2 siRNA. $^{**}P < 0.01$ versus the NG group; $^{##}P < 0.01$ versus the HG group; $^{\Delta\Delta}P < 0.01$ versus the HG+CA group. Data are the means $\pm$ SEM from 4 independent experiments. The scale bar indicates 10 μ m.

translocation, inducing the expression of target proteins HO-1, NQO1, CAT and GPx-1 in high glucose-treated HUVECs and thus also decreasing the level of nitrotyrosine. In high glucose-treated HUVECs, CA treatment attenuated ROS generation and prevented NO depletion, but

these effects were blocked by Nrf2 siRNA, indicating that CA prevented high glucose-induced endothelial dysfunction through endogenous Nrf2 signal activation.

Diabetes is characterized by hyperglycemia and the development of diabetic vascular complications. The primary causative factor leading to the pathophysiologic alterations in the diabetic vasculature is exposure to a high blood glucose level [17]. In spite of the significant developments in anti-diabetic therapy, diabetic vascular complications continue to be seriously detrimental [17]. Therefore, it is critical to pursue novel strategies for preventing the vascular complications associated with diabetes.

CA is a spice compound in cinnamon that has been widely used as a component in perfumes, as a fungicide, and as a flavoring agent in foodstuffs, such as chewing gum, ice cream, candy and beverages [18]. Increasing evidence has demonstrated that CA has many pharmacological activities, including anti-hyperglycemic, anti-oxidative stress, anti-cancer, cardiovascular protective, etc. [9, 19, 20], indicating that CA may play a protective role in diabetic vascular complications, but its direct effect on endothelial dysfunction induced by high glucose was unknown.

The accelerated degradation of NO by ROS is most likely the major mechanism that limits NO bioavailability in states of cardiovascular disease [21]. In our study, we first found that the endothelial ROS level increased and the NO level decreased after high glucose treatment for 24 h; these changes may be responsible for the impairment of endothelium-dependent relaxation. Pretreatment with CA attenuates the ROS generation and prevented the decrease of NO, thus preserving endothelium-dependent relaxation in mice aortas under hyperglycemic conditions. These results showed the antioxidant properties and endothelial protective effects of CA under high glucose conditions.

We next asked how CA protected the endothelium under hyperglycemic conditions. Previous studies indicated that dietary cinnamon-derived CA activates the Nrf2-dependent antioxidant response in human epithelial colon cells and may therefore represent a chemopreventive dietary factor targeting colorectal carcinogenesis [22]. CA enhances Nrf2 nuclear translocation to upregulate phase II detoxifying enzyme expression in HepG2 cells [11]. These studies indicate that CA is an Nrf2 activator. However, whether CA also acts as an Nrf2 activator in the endothelium under hyperglycemia is unknown. In our study, Western blotting indicated that CA increased Nrf2 expression and promoted Nrf2 nuclear translocation in HUVECs exposed to high glucose. These results indicated that CA also acts as an Nrf2 activator in high glucose-treated HUVECs.

Nrf2, first cloned and characterized by its ability to bind to the NF-E2/AP-1 repeat in the promoter of the beta-globin gene, is ubiquitously expressed in many organs as a transcriptional activator of phase II detoxifying genes by binding to antioxidant responsive element (ARE) sequences [23].

Many cardiovascular diseases, including diabetic vascular complications, are associated with a failure of defenses against oxidative stress-induced cellular damage and/or death, leading to organ dysfunction [24]. It is well documented that Nrf2 plays a protective role in cardiovascular diseases such as atherosclerosis, hypertension, and heart failure due to its antioxidant properties [25]. Nrf2 activation depends on Kelch ECH Associating Protein 1, a cytoskeletal protein that binds to actin filaments and Nrf2 to prevent the nuclear translocation of Nrf2, thus acting as a transcriptional repressor during basal conditions [25].

HO-1 is well known for its cytoprotective effects against oxidative injuries and inflammation *in vitro* and *in vivo* [26]. The overexpression of HO-1 plays a protective role in myocardial ischemia/reperfusion injury in diabetic mice [27]. The treatment of endothelial cells with high glucose for 7 days decreased the HO-1 activity and cell viability [28]. HO-1 up-regulation in diabetic rats led to reduced ROS production and decreased endothelial cell sloughing [29]. It has been suggested that inducible NQO1, which is highly and constitutively expressed in cardiovascular cells, may act as a superoxide scavenger [30]. Thus, the upregulation of HO-1 and NQO1 in response to high glucose provides an effective endogenous antioxidant defense mechanism in diabetes and other vascular diseases [6]. We next explored whether CA could further increase the expression of the downstream

proteins HO-1 and NQO1. Immunoblotting confirmed that CA increased both HO-1, NQO1, CAT and GPx-1 expression in HUVECs under high glucose conditions, and these effects may be responsible for the decreased nitrotyrosine levels.

Finally, we investigated whether the effects of CA on endothelial cells was associated with Nrf2. DHE and DAF-2DA staining demonstrated that CA has direct effects on ROS and NO levels in ECs under high glucose, and these effects were blocked by Nrf2 siRNA. These results confirmed that the induction of ROS generation by CA under high glucose conditions was mediated by Nrf2 signal pathway activation.

In summary, this study shows that treatment with CA can prevent high glucose-induced endothelial dysfunction. Our mechanistic evidence suggests that this vascular benefit is likely to result from an enhancement of Nrf2 expression and nuclear translocation, which leads to the up-regulation of both HO-1, NQO1, CAT and GPx-1 expression and attenuates the generation of ROS and nitrotyrosine levels in ECs under high glucose conditions. Our findings provide new insight into the direct role of CA in diabetes vascular dysfunction. Nrf2 activation by CA may represent a promising intervention in diabetic patients who are at risk for vascular complications.

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Disclosure Statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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