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# Astaxanthin alleviates oxidative stress insults-related derangements in human vascular endothelial cells exposed to glucose fluctuations

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ABSTRACT

Glycemic fluctuations may play a critical role in the pathogenesis of diabetic complications, such as cardiovascular disease. We investigated whether the oxycarotenoid astaxanthin can reduce the detrimental effects of fluctuating glucose on vascular endothelial cells. Human umbilical venous endothelial cells were incubated for 3 days in media containing 5.5 mM glucose, 22 mM glucose, or 5.5 mM glucose alternating with 22 mM glucose in the absence or presence of astaxanthin or N-acetyl-L-cysteine (NAC). Constant high glucose increased reactive oxygen species (ROS) generation, but such an effect was more pronounced in fluctuating glucose. This was associated with up-regulated $p22^{phox}$ expression and down-regulated peroxisome proliferator activated receptor-γ coactivator (PGC-1α) expression. Astaxanthin inhibited ROS generation, $p22^{phox}$ up-regulation, and PGC-1α down-regulation by the stimuli of glucose fluctuation. Fluctuating glucose, but not constant high glucose, significantly decreased the endothelial nitric oxide synthase (eNOS) phosphorylation level at Ser-1177 without affecting total eNOS expression, which was prevented by astaxanthin as well as by the anti-oxidant NAC. Transferase-mediated dUTP nick end labeling (TUNEL) showed increased cell apoptosis in fluctuating glucose. Glucose fluctuation also resulted in up-regulating gene expression of pro-inflammatory mediators, interleukin-6 and intercellular adhesion molecule-1. These adverse changes were subdued by astaxanthin. The phosphorylation levels of c-Jun N-terminal kinase (JNK) and p38 were significantly increased by glucose fluctuations, and astaxanthin significantly inhibited the increase in JNK and p38 phosphorylation. Taken together, our results suggest that astaxanthin can protect vascular endothelial cells against glucose fluctuation by reducing ROS generation.


## 1. Introduction

Individuals with diabetes mellitus are at an increased risk for cardiovascular disease which can eventually be disabling or even life-threatening [1]. Chronic hyperglycemia, which is contributed by fasting and postprandial hyperglycemia, plays a central role in developing diabetic cardiovascular complications, and the benefit of glycemic control in reducing the risk for cardiovascular disease has been established [2]. However, the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial has revealed that intensive glycemic control can increase all-cause mortality more than standard therapy [3], and the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) trial has demonstrated that intensive glucose-lowering strategy has no significant effect in reducing macrovascular disease [4]. In the meantime, fluctuations in blood glucose levels that can potentially occur in inadequate glycemic management with insulin or oral antidiabetics have also been identified as a risk for cardiovascular events [5,6]. Furthermore, diabetic individuals may experience fluctuations in blood glucose levels with a habitual diet that is high in concentrated sweets [7].

Vascular endothelial cells have emerged as a key regulator of vascular homeostasis, in that they have not merely a barrier function but also produce and secrete mediators that regulate a wide range of physiological and pathological processes, including vasomotor tone, angiogenesis, inflammation, and coagulation [8–10]. A growing body of substantial evidence demonstrates that endothelial dysfunction underlies the basic pathophysiology of diabetic vascular disorders [10–12]. In clinical practice, vascular endothelial function has been portrayed to be impaired by fluctuations in blood glucose levels in type 2 diabetic patients [13]. Intriguingly, intermittent high glucose appears to be more harmful

*Abbreviations:* CM-$H_2$DCFPA, 5-(and-6)-chloromethyl-2′7′-dichlorofluorescein diacetate; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical venous endothelial cell; ICAM-1, intercellular adhesion molecule-1; JNK, c-Jun N-terminal kinase; IL-6, interleukin-6; MAPK, mitogen-activated protein kinase; Mn-SOD, manganese-dependent superoxide dismutase; NAC, N-acetyl-L-cysteine; NO, nitric oxide; NOx, nitrite and nitrate; PGC-1α, peroxisome proliferator activated receptor-γ coactivator 1α; ROS, reactive oxygen species; TUNEL, deoxynucleotide transferase-mediated dUTP nick end labeling.

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than constant high glucose medium for human vascular endothelial cells. It has been shown that periodic exposure of human vascular endothelial cells to high glucose results in elevated apoptosis [14–16], up-regulated expression of adhesion molecules [17,18], and accelerated senescence [19,20].

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a non-provitamin A carotenoid classified as a xanthophyll and is found in high amounts in the red pigment of crustacean shells (e.g., crabs, shrimp), salmon, trout, and asteroidean [21,22]. It has been demonstrated that astaxanthin displays a wide variety of biological activities, including anti-oxidative, anticancer, and anti-inflammatory effects [22]. Given such biological features of this naturally occurring carotenoid, astaxanthin may serve as a vascular endothelial protecting agent. Recent studies using animal models of diabetes and hypertension suggest that astaxanthin can alleviate endothelial dysfunction under these pathological conditions [23,24].

In the present study, we tested the hypothesis that astaxanthin can reduce the detrimental effects of fluctuating glucose on vascular endothelial cells. We observed that fluctuating glucose led to highly increased generation of reactive oxygen species (ROS), decreased basal activity of endothelial nitric oxide synthase (eNOS), increased endothelial cell apoptosis, and up-regulated gene expression of pro-inflammatory mediators in human vascular endothelial cells. We found that treatment with astaxanthin protected endothelial cells from the detrimental changes by glucose fluctuation.

## 2. Materials and methods

### 2.1. Cell culture

Human umbilical venous endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany) and Lonza (Basal, Switzerland) and were cultured in endothelial cell growth medium-2 (Takara Bio, Otsu, Japan) until the start of the experiment. HUVECs were cultured at 37 °C under a humidified atmosphere containing 5% $CO_2$ and 95% air. According to our previous studies [25,26], four- to seven-passage subconfluent cells were used in the experiments. Cells were harvested at 70–80% confluence and seeded into plates with variable sizes. Then, they were exposed to the experimental condition for 3 days. Namely, they were grouped as follows: (1) constant normal glucose medium (5.5 mM); (2) constant high glucose medium (22 mM); and (3) alternating normal and high glucose media every 12 h. During the experimental period, cells were incubated in modified endothelial cell growth medium-2 (Lonza) that contained 2% fetal bovine serum but lacked insulin-like growth factor-1 in order to exclude the insulin effect. In the constant normal or high glucose group, each fresh medium was given every 12 h. In the intermittent high glucose group, cells were placed under the high glucose condition before harvesting. Mannitol was used to rule out the effect of osmotic pressure [25]. When cells were treated with astaxanthin or N-acetyl-L-cysteine (NAC), these agents were added at the start of high glucose exposure and remained present throughout the experiment.

### 2.2. Imaging of intracellular ROS by fluorescence microscopy

Intracellular ROS generation was measured by a confocal microscopy as previously described [26]. We loaded endothelial cells with the fluorescence probe 5-(and-6)-chloromethyl-2′7′-dichlorofluorescein diacetate, acetyl ester (CM-$H_2$DCFDA; Invitrogen, Carlsbad, CA, USA) to a final concentration of 10 μM for 45 min in HuMedia-EB2 medium (Kurabo, Osaka, Japan) and then placed in culture medium. Imaging was conducted using BIOREVO BZ-9000 (Keyence, Osaka, Japan). ImageJ was used for image analysis. $H_2O_2$ (200 μM) served as a positive control. The level of intracellular ROS was determined by comparing the fluorescence intensities of treated cells with those of control cells.

### 2.3. Western blot analysis

Cells were grown in 100-mm dish, harvested, and lysed in 300 μl of RIPA buffer (25 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) containing protease inhibitor cocktail on ice. The lysates were centrifuged at 18,000 ×*g* for 10 min at 4 °C and the resulting supernatants were collected. The proteins in the supernatant were measured using BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Immunoblotting was performed as described in our previous reports [25,27]. Samples (30–50 μg of protein) were run on 10% SDS-polyacrylamide gel and electrotransferred to polyvinylidene difluoride filter membrane. The membrane was blocked for 60 min at room temperature in PBS containing 1% bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) or 3% skimmed milk (Nakalai Tesque, Kyoto, JAPAN), or in Odyssey blocking buffer (LI-COR Bioscience, Lincoln, NE, USA) followed by overnight incubation with primary antibody at 4 °C. Primary antibody detection was performed with horseradish peroxidase-conjugated or IRDye®-labeled secondary antibodies. Binding of the antibody was detected by an ImmunoStar Zeta (Wako Pure Chemical, Osaka, Japan) and levels of protein expression were quantitated by a luminoimage LAS-4000 analyzer (Fuji Film, Tokyo, Japan). Fluorescent of IR-Dye was analyzed by Odyssey CLx Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA).

The following antibodies, which are commercially available, were used: anti-human $p22^{phox}$ rabbit polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human Nox4 goat monoclonal antibody (1:500; Santa Cruz Biotechnology), anti-human manganese-dependent superoxide dismutase (Mn-SOD) rabbit monoclonal antibody (1:500; Millipore, Billerica, MA, USA), anti-human peroxisome proliferator activated receptor-γ coactivator 1α (PGC-1α) rabbit polyclonal antibody (1:1000; Santa Cruz Biotechnology), anti-human eNOS mouse monoclonal antibody (1:1000; BD Biosciences, San Jose, CA, USA), anti-human phospho-eNOS (Ser-1177) mouse monoclonal antibody (1:1000; BD Biosciences), anti-human phospho-eNOS (Thr-495) rabbit monoclonal antibody (1:1000; BD Biosciences), anti-human extracellular signal-regulated protein kinase 1/2 (ERK1/2) mouse monoclonal antibody (1:1000; Cell Signaling, Danvers, MA, USA), anti-human phospho-ERK1/2 (Thr-202/Tyr-204) rabbit monoclonal antibody (1:1000; Cell Signaling), anti-human c-Jun N-terminal kinase (JNK) rabbit monoclonal antibody (1:1000; Cell Signaling), anti-human phospho-stress-activated protein kinase (SAPK)/JNK (Thr-183/Tyr-185) mouse monoclonal antibody (1:1000; Cell Signaling), anti-human p38 rabbit monoclonal antibody (1:1000; Cell Signaling), anti-human phospho-p38 (Thr-183/Tyr-182) mouse monoclonal antibody (1:1000; Cell Signaling). Anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) chicken polyclonal antibody (1:1500; Millipore) and anti-human β-actin rabbit polyclonal antibody (1:1000; Bioss, Woburn, MA, USA) were used as a loading control.

### 2.4. RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells with Sepazol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) was used for the reverse transcription reaction, and real-time PCR analyses were performed using SYBR Premix Ex Taq (Tli RNaseH Plus), ROX plus (Takara Bio, Ohtsu, Japan). The p22-phox primer sequences were 5′-GTACTTTGGTGCCTACTCCA-3′ (sense) and 5′-CGGCCCGAACATAGTAATCC-3′ (antisense), the Nox4 primer sequences were 5′-AGTCAAACAGATGGGATA-3′ (sense) and 5′-TGTCCC ATATGAGTTGTT-3′ (antisense), the Mn-SOD primer sequences were 5′-TTTCAATAAGGAACGGGGACAC-3′ (sense) and 5′-GTGCTCCCACAC ATCAATCC-3′ (antisense), the PGC-1α primer sequences were 5′-GTCA CCACCCAAATCCTTAT-3′ (sense) and 5′-ATCTACTGCCTGGAGACCTT-3′ (antisense), the intercellular adhesion molecule-1 (ICAM-1) primer sequences were 5′-AGCCAACCAATGTGCTATTCAAAC-3′ (sense) and 5′-CACCTGGCAGCGTAGGGTAA-3′ (antisense), and the interleukin-6 (IL-6)