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Protection against Oxidative Stress, Inflammation, and Apoptosis of High-Glucose-Exposed Proximal Tubular Epithelial Cells by Astaxanthin

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Astaxanthin is a carotenoid with powerful antioxidant properties that exists naturally in various plants, algae, and seafood. The purpose of the present study is to examine the protective action of astaxanthin against high-glucose-induced oxidative stress, inflammation, and apoptosis in proximal tubular epithelial cells (PTECs). To assess the efficacy of astaxanthin, several key markers and activities were measured, including lipid peroxidation, total reactive species (RS), superoxide ($^{\bullet}O_2$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$), as well as expressions of inflammatory proteins, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-κB) nuclear translocation, and levels of Bcl2/Bax protein. Results showed that astaxanthin effectively suppressed lipid peroxidation, total RS, $^{\bullet}O_2$, $NO^{\bullet}$, $ONOO^-$, iNOS and COX-2 protein levels, NF-κB nuclear translocation, and pro-apototic Bax, whereas it increased anti-apoptotic Bcl2 protein levels. On the basis of these findings, it was concluded that in PTECs, astaxanthin has a protective efficacy against several deleterious effects caused by high glucose exposure and proposed that astaxanthin should be explored further as a potential antidiabetic remedy for the treatment of diabetic nephropathy.

KEYWORDS: Astaxanthin; oxidative stress; inflammation; diabetes

INTRODUCTION

Antioxidants from natural sources have become increasingly important as therapeutic agents against oxidative stress and inflammation-related diseases such as diabetes (*1*). Astaxanthin is a red-orange carotenoid pigment (see the chemical structure in **Figure 1**) that occurs naturally in a wide variety of living organisms and has a potent antioxidant capacity (*2*). Astaxanthin is shown to possess various beneficial properties including anticancer, anti-inflammatory, immunomodulatory, and neuroprotective activities (*2*) and the prevention of diabetic nephropathy in diabetic db/db mice (*3*). At the cellular level, astaxanthin protects mitochondrial redox homeostasis against oxidative stress (*4*) while inhibiting nitric oxide production and inflammatory gene expression by suppressing I (κ)B kinase-dependent NF-κB (*5*). Dietary astaxanthin supplementation, in combination with α-tocopherol, shows an inhibitory effect on oxidative stress in diabetic ODS rats (*6*). Recently, Manabe et al. (*7*) suggested the beneficial effect of astaxanthin may be related to its ability to suppress mitochondrial oxidative alterations. However, the precise mechanisms underlying the protective effects against diabetic nephropathy remain unresolved to date.

Oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) leads to cellular damage, which is a causative factor in chronic degenerative diseases including diabetes (*8*). Diabetes mellitus is characterized by hyperglycemia, an abnormal elevation in the blood glucose level that also is associated with oxidative stress (*6*). Under diabetic conditions, many damaged molecules including DNA, lipid peroxidation products, and advanced glycation end products (AGEs) eventually lead to pathological diabetic complications including nephropathy, retinopathy, and neuropathy (*6*, *9*).

It is well-known that poor glycemic regulation leads to the development of diabetic nephropathy, but the mechanisms underlying high-glucose-exposed cell and tissue injury are yet to be elucidated.

Additionally, it has been shown that proximal tubular epithelial cells (PTECs) are a primary target of hyperglycemia and that the chronic exposure of PTECs to increased blood glucose levels leads to the tubulointerstitial changes seen in overt diabetic nephropathy (*10*). It has been reported that high glucose was responsible for the initiation and progression of diabetic nephropathy and caused cytotoxicity in PTECs (*11*, *12*).

In recent years, many investigators have used the high-glucose-exposed renal cell model to study the cellular and molecular mechanisms of diabetic nephropathy (*10*−*12*). Most recently, a close association was suggested between hyperglycemia and oxidative stress and diabetic complications (*8*). Our group (*9*) and that of Rivero et al. (*13*) also have reported on the strong relationship between oxidative stress and inflammation in the pathologies of diabetes. Furthermore, it has been reported that hyperglycemia selectively provokes apoptosis in tubule and

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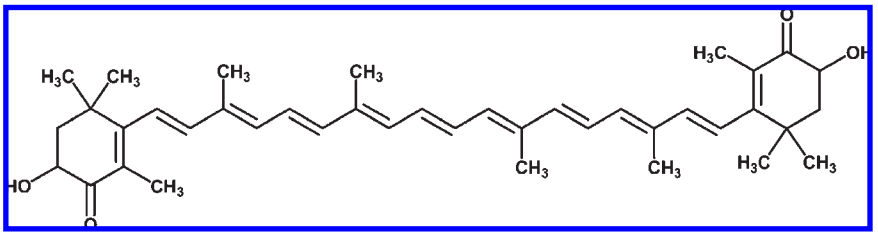

Figure 1. Chemical structure of astaxanthin.

endothelial cells (*11*) and that members of the Bcl2 family of proteins regulate the occurrence of apoptotic cell death (*14*). Bcl2 is known as an upstream effector molecule in the apoptotic pathway and has been identified as a strong inhibitor of apoptosis. Bcl2 forms a heterodimer with the apoptotic protein Bax, thereby neutralizing its apoptotic effects. Thus, change in the ratio of Bax/Bcl2 is a decisive factor that plays an important role in determining whether cells will undergo apoptosis (*14*). In this study, high-glucose-exposed PTECs were used to investigate the possible beneficial role of astaxanthin on diabetic nephropathy, oxidative stress, inflammation, and apoptosis.

To verify the effects of astaxanthin against oxidative stress, measurements of lipid peroxidation, total reactive species (RS), superoxide ($^{\bullet}O_2$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) were performed. Additionally, to elucidate the mechanisms related to inflammation, the expression of key inflammatory proteins, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and nuclear factor κB (NF-κB) nuclear translocation were evaluated. We also assessed the effects of astaxanthin on anti-apoptotic Bcl2 protein levels and pro-apoptotic Bax protein levels.

MATERIALS AND METHODS

Materials. Astaxanthin was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) was purchased from GIBCO (Grand Island, NY), and fetal calf serum (FCS) was purchased from Life Technologies Inc. (Grand Island, NY). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), dihydrorhodamine 123 (DHR 123), and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG were purchased from Sigma-Aldrich Co. Monoclonal anti-cyclooxygenase-1 (COX-1), monoclonal anti-COX-2, monoclonal anti-NOS and anti-Bcl2 antibodies, and polyclonal anti-NF-κB, anti-Bcl2, anti-Bax, and peroxidase-labeled secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were of the highest commercial grade available.

Cell Culture. The porcine proximal tubular epithelial cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM/F-12 medium supplemented with 5% FCS in a humidified atmosphere containing 5% CO_2 in air at 37 °C.

Lipophilic astaxanthin was dissolved in dimethyl sulfoxide (DMSO) and added to the cells at a final concentration of 0.5% (v/v). Control vehicle (0.5% DMSO, v/v) showed no significant cytotoxicity. All subsequent procedures were carried out under these conditions, and all of the experiments were determined in triplicate and repeated three times to ensure reproducibility.

Assessments of Cell Viability and Morphology. The cell viability assay was performed according to the method of Carmichael et al. (*15*) using MTT. PTECs were seeded at 2×10^4/mL in 96-well plates and preincubated for 24 h. The cells were pretreated with 5 mM glucose for 24 h before treatment with 30 mM glucose and astaxanthin at the same time.

A 50 μL aliquot of MTT solution (1 mg/mL) was added to each well of a 96-well culture plate (n = 6/group) and incubated for 4 h at 37 °C, and the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 μL of DMSO, and the absorbance at 540 nm of each well was read using a model 3550-UV microplate reader (Bio-Rad, Tokyo, Japan). For the morphology test, cells were grown on 6-well plates, treated with glucose and astaxanthin for 24 h, and photographed.

Estimation of Total RS Generation. Total RS generation was assayed in culture supernatant (*16*). Twenty-five millimolar DCFH-DA was added to incubation media, and changes in fluorescence were estimated at an excitation wavelength of 486 nm and an emission wavelength of 530 nm for 30 min.

Assay of $^{\bullet}O_2$ Level. The generation and inhibition of $^{\bullet}O_2$ were assessed following the method described by Ewing and Janero (*17*). The $^{\bullet}O_2$-scavenging activity was estimated by assessing the decrease in the ratio of the reduction of nitro blue tetrazolium (NBT). Culture supernatant was added to the reaction buffer (50 mM PBS with 125 μM EDTA, 62 μM NBT, and 98 μM NADH) containing 33 μM 5-methylphenazium methyl sulfate. The absorbance at 540 nm, as an index of NBT reduction, was estimated after 5 min.

Measurement of $NO^{\bullet}$ Level. $NO^{\bullet}$ production was determined by assessing the accumulation of nitrite in the conditioned medium according to the Griess assay. In short, 100 μL of culture supernatant was allowed to react with 100 μL of Griess reagent (*18*) and then incubated at room temperature for 5 min. The optical density at 540 nm of the samples was estimated using a microplate reader.

Determination of $ONOO^-$ Level. $ONOO^-$-dependent oxidation of DHR 123 to rhodamine 123 was evaluated on the basis of the method described by Kooy et al. (*19*). Samples were added to the rhodamine buffer (pH 7.4) containing 6.25 μM DHR 123 and 125 μM diethylenetriaminepentaacetic acid and incubated for 5 min at 37 °C. The absorbance was measured at 500 nm, which is the absorbance of rhodamine 123.

Estimate of Lipid Peroxidation by TBARS. The concentration of thiobarbituric acid reactive substances (TBARS) was estimated according to the method of Buege and Aust (*20*) with slight modification. This method involves the creation of a colored complex between the products of lipid peroxidation and thiobarbituric acid at a temperature of 100 °C in an acidic environment. The maximum absorption of this complex occurs at a wavelength of 532 nm.

Immunochemical Evidence on NF-κB Nuclear Translocation. After incubation and treatment, PTECs were fixed with 3.7% paraformaldehyde for 30 min at 4 °C, washed with PBS, and permeabilized with 0.2% Triton X-100 for 30 min at 4 °C. The cells were washed with PBS, blocked with 2% bovine serum albumin (BSA) for 1 h, and treated with anti-NF-κB antibody for 2 h at 4 °C. The anti-NF-κB stained cells were washed with PBS, incubated with FITC-conjugated anti-rabbit IgG for 1 h at 4 °C, washed with PBS, and then stained with DAPI for 5 min at room temperature. The cells were washed twice more with PBS, and analysis was performed using a fluorescent microscope.

Protein Extraction and Western Blotting. Total cell lysates were produced by lysing cells in an extraction buffer [25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1% nonidet P-40 (NP-40), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 50 μL of protease inhibitor cocktail]. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). For Western blot analysis, aliquots containing 34 μg of protein were separated by SDS−polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were subjected to immunoblot analysis, and proteins were visualized by the enhanced chemiluminescence method (Amersham Pharmacia Biotech U.K. Limited, Bucks, U.K.).

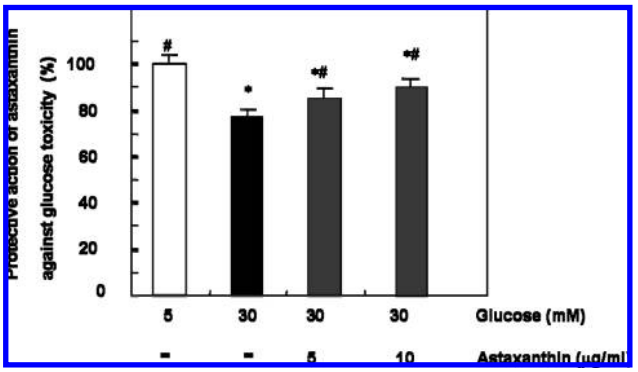

Figure 2. Protective action of astaxanthin against glucose toxicity. Cells were treated with 5−10 (μg/mL) astaxanthin and were evaluated by MTT assay. Data are represented as mean ± SEM of at least three determinations. ∗, $p < 0.05$, and ∗∗, $p < 0.01$, compared with 5 mM glucose-treated values; #, $p < 0.05$, and ##, $p < 0.01$, compared with 30 mM glucose-treated values.

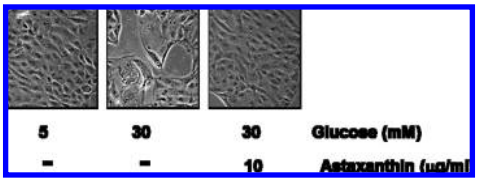

Figure 3. Effect of astaxanthin on morphological changes. The cells were observed under light microscopy. Magnification: ×200.

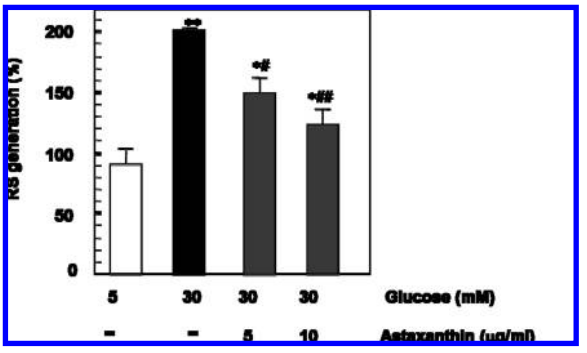

Figure 4. Modulation of total RS generation by astaxanthin. Results are expressed as the mean ± SEM. ∗, $p < 0.01$, and ∗∗, $p < 0.001$, compared with 5 mM glucose-treated values; #$p < 0.01$, and ##, $p < 0.001$, compared with 30 mM glucose-treated values.

Statistical Analysis. All of the experiments were determined in triplicate and repeated three times to ensure reproducibility. The effect of each parameter was examined using the one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test, and those at $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

In the present study, we investigated the efficacy of astaxanthin on diabetic nephropathy, utilizing an in vitro model of high-glucose-treated PTECs. We believe we show, for the first time, that astaxanthin ameliorated oxidative stress, inflammation, and apoptosis in these cells.

As a preliminary study, the putative properties of astaxanthin were examined in relation to cell viability and morphology in PTECs. **Figure 2** shows that the 30 mM glucose-treated cells had significantly reduced cell viability as compared to the 5 mM glucose-treated cells. However, treatment with 5 and 10 μg/mL astaxanthin with the high glucose treatment significantly enhanced cell viability. The morphological changes in the 30 mM glucose-treated cells and protection of astaxanthin are shown in **Figure 3**. Therefore, these data clearly show that astaxanthin is effective against high-glucose-induced cytotoxicity.

Table 1. Effects of Astaxanthin on $^{\bullet}O_2$, NO$^{\bullet}$, and $ONOO^-$ Levels in Glucose-Treated Cells[a]

glucose (mM)	astaxanthin (μg/mL)	$^{\bullet}O_2$ (%)	NO$^{\bullet}$ (%)	$ONOO^-$ (%)
5		100 ± 0.8	100 ± 3.7	100 ± 0.4
30		249 ± 1.0 **	265 ± 1.8 **	295 ± 0.3 **
30	5	145 ± 0.5 *,#	155 ± 1.13 *,#	183 ± 3.43 *,#
30	10	92 ± 9.0 ##	122 ± 1.90 **	137 ± 2.15 *,##

[a] Results are expressed as the mean ± SEM. Significance: *, $p < 0.01$; **, $p < 0.001$, vs 5 mM glucose-treated values; #, $p < 0.01$, and ##, $p < 0.001$, vs 30 mM glucose-treated control values.

To understand the underlying actions of the antidiabetic effect of astaxanthin in relation to its antioxidative actions, we evaluated the effect of astaxanthin on total RS, $ONOO^-$, $^{\bullet}O_2$, and NO$^{\bullet}$. As shown in **Figure 4**, the astaxanthin-treated cells had significantly suppressed RS generation. $^{\bullet}O_2$, NO$^{\bullet}$, and $ONOO^-$ levels were augmented in the 30 mM glucose-treated cells (**Table 1**); however, astaxanthin showed scavenging activities against these RS. Our current findings are in agreement with previous studies (*9*, *21*), confirming the notion of the role of oxidative stress in the pathologies of diabetes.

The salient finding of our present study is that the suppression of total RS, specifically, $^{\bullet}O_2$, NO$^{\bullet}$, and $ONOO^-$, is likely the crucial, key factor in the antioxidative ability of astaxanthin in high-glucose-treated PTECs, as shown in **Figure 4** and **Table 1**. These data revealed the broad and effective scavenging actions of astaxanthin as indicators of its overall antioxidant property. The scavenging ability of $ONOO^-$ by astaxanthin is especially noteworthy because of its well-known damaging power, as recently shown by diabetic nephropathy injury (*22*).

The broad antioxidative action of astaxanthin was also shown by its ability to suppress lipid peroxidation. Lipid peroxidation is known to increase under diabetic conditions (*23*). **Figure 5** shows that TBARS levels were significantly suppressed in the astaxanthin-treated group, indicating lipid peroxidation inhibition by astaxanthin. Thus, this ability of astaxanthin to thwart lipid peroxidation is likely a strong contributing factor to its antihyperglycemic activity in diabetes.

Several recent studies implicate the inflammatory process in the pathogenesis of diabetic nephropathy (*9*, *14*, *24*). On the basis of the broad, effective antioxidative action of astaxanthin, we expected that astaxanthin might have an anti-inflammatory efficacy. **Figure 7** shows the expected anti-inflammatory property of astaxanthin on the suppression of COX-2 expression. Not only did our data on several proinflammatory markers, that is, NF-κB activity, COX-2, and iNOS, support this notion, but also the data revealed that the high-glucose-induced overexpressions of COX-2, iNOS, and nuclear translocation of NF-κB were well attenuated by astaxanthin treatment. These findings strongly indicate the possibility that astaxanthin acts as an anti-inflammatory agent, thereby exerting an additional protective role against diabetes or hyperglycemia.

Although recent papers (*25*, *26*) have shown that in various experimental models of renal injury NF-κB is activated in tubules and glomeruli, the biological significance of NF-κB activation in diabetic renal injury has not been fully shown. Our data in **Figure 6** illustrate the effect of astaxanthin on the nuclear translocation of NF-κB by 30 mM glucose treatment that was blocked by 10 μg/mL astaxanthin. To ascertain the integrity of nuclei and cells, we performed DAPI staining (**Figure 6**). The

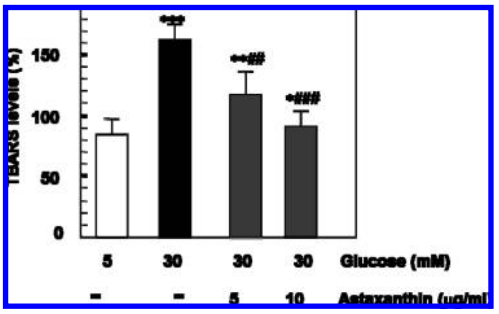

Figure 5. Inhibitory effect of astaxanthin on TBARS levels. Results are expressed as the mean ± SEM of at least three determinations. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, compared with 5 mM glucose-treated values; ##, $p < 0.01$, and ###, $p < 0.001$, compared with 30 mM glucose-treated values.

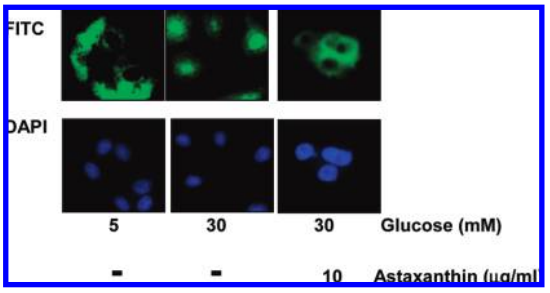

Figure 6. Effect of astaxanthin on the nuclear translocation of NF-κB. Cells were incubated with glucose and astaxanthin for 48 h. Fixed cells were double immunostained for NF-κB (green) and DAPI (blue). Magnification: ×400.

high-glucose-induced NF-κB nuclear translocation was attenuated in astaxanthin-treated PTECs, which is in line with NF-κB nuclear translocation by anti-inflammatory reagents (*27*), and supported by our previous report that the suppression of the NF-κB pathway is an important factor underlying inflammation-related diabetes (*9*).

It also has been shown that apoptosis in PTECs plays a significant role in acute renal failure as hyperglycemia enhances apoptosis (*28*). In our study, the cells treated with 30 mM glucose showed decreased anti-apoptotic Bcl2 protein expression, but those treated with astaxanthin showed enhanced Bcl2 expression (**Figure 7**). On the other hand, pro-apoptotic Bax expression was increased in the 30 mM glucose-treated cells but suppressed by astaxanthin treatment (**Figure 7**). Our current results on increased apoptosis by high glucose exposure are consistent with a previous study by Allen et al. (*11*), showing that high-glucose-induced oxidative stress results in apoptosis in PTECs. It would be interesting to surmise that the present data showing the ability of astaxanthin to attenuate hyperglycemia-induced apoptosis in PTECs stems from the inhibition of oxidative stress. Our results also are consistent with the previous findings (*29*) that antioxidants modulate the Bcl2/Bax proteins, indicating various beneficial effects in preventing tubulointerstitial injury in diabetic nephropathy.

Considering the beneficial actions of astaxanthin are intracellular events, it would be interesting and important to discuss something about the permeability of this lipophilic astaxanthin. Pashkow et al. (*30*) reported that localization of astaxanthin in the membrane lipid bilayer is likely responsible for its antioxidative properties and biological benefits. Manabe et al. (*7*) reported that the quantitative analysis of astaxanthin in mitochondria and cytoplasm shows mitochondrial accumulation in human mesangial

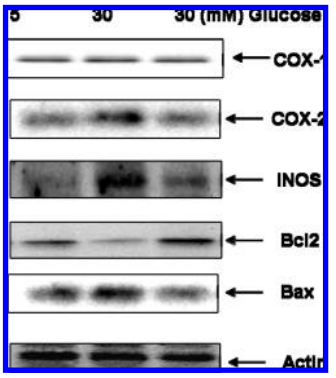

Figure 7. Changes in iNOS, COX-2, Bcl2, and Bax expressions. After 48 of incubation with glucose and astaxanthin, cells were lysed and 30 μg of cell lysates was used for Western blotting analysis.

cells. Although we have no quantitative information on the intracellular astaxanthin level in the present study, based on the afore-cited work, we expect that sufficient amounts of astaxanthin were intracellularly localized to exert its protection against oxidative stress, inflammation, and apoptosis.

In summary, our present work demonstrated that astaxanthin ameliorates an overall oxidative stress state induced by high glucose by its ability to suppress lipid peroxidation, total RS generation, $ONOO^-$, $^{\bullet}O_2$, and $NO^{\bullet}$. Additionally, the data show that astaxanthin suppresses the inflammatory process by modulating COX-2, iNOS, and NF-κB nuclear translocation. Furthermore, astaxanthin was shown to uphold anti-apoptotic Bcl2 protein levels while suppressing pro-apoptotic Bax protein levels.

On the basis of all the evidence, we propose that the modulation of oxidative stress, inflammation, and apoptosis is the likely mechanism underlying the beneficial role of astaxanthin on diabetic nephropathy in high-glucose-treated PTECs. Consequently, we conclude from these studies that astaxanthin could be an effective, functional supplement as an antidiabetic phytochemical. Further investigations are needed to delineate the molecular properties underlying the protective action of astaxanthin on cellular homeostasis and signal transduction pathways.

ABBREVIATIONS USED

AGEs, advanced glycation end products; COX, cyclooxygenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate; DCFH-DA, 2,7-dichlorofluorescein diacetate; DHR 123, dihydrorhodamine 123; DMEM/F-12, Dulbecco's modified Eagle medium/nutrient mixture F-12; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NBT, nitro blue tetrazolium; NF-κB, nuclear factor-kappa B; $NO^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; NP-40, nonidet P-40; $^{\bullet}O_2$, superoxide; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PTECs, proximal tubular epithelial cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; RS, reactive species; TBARS, thiobarbituric acid reactive substances.

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