Phycocyanin and phycocyanobilin from *Spirulina platensis* protect against diabetic nephropathy by inhibiting oxidative stress

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1Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 2Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka, Japan; and 3NutriGuard Research, Incorporated, Encinitas, California

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Zheng J, Inoguchi T, Sasaki S, Maeda Y, McCarty MF, Fuji M, Ikeda N, Kobayashi K, Sonoda N, Takayanagi R. Phycocyanin and phycocyanobilin from *Spirulina platensis* protect against diabetic nephropathy by inhibiting oxidative stress. *Am J Physiol Regul Integr Comp Physiol* 304: R110–R120, 2013. First published October 31, 2012; doi:10.1152/ajpregu.00648.2011.—We and other investigators have reported that bilirubin and its precursor biliverdin may have beneficial effects on diabetic vascular complications, including nephropathy, via its antioxidant effects. Here, we investigated whether phycocyanin derived from *Spirulina platensis*, a blue-green algae, and its chromophore phycocyanobilin, which has a chemical structure similar to that of biliverdin, protect against oxidative stress and renal dysfunction in *db/db* mice, a rodent model for Type 2 diabetes. Oral administration of phycocyanin (300 mg/kg) for 10 wk protected against albuminuria and renal mesangial expansion in *db/db* mice, and normalized tumor growth factor-β and fibronectin expression. Phycocyanin also normalized urinary and renal oxidative stress markers and the expression of NAD(P)H oxidase components. Similar antioxidant effects were observed following oral administration of phycocyanobilin (15 mg/kg) for 2 wk. Phycocyanobilin, bilirubin, and biliverdin also inhibited NADPH dependent superoxide production in cultured renal mesangial cells. In conclusion, oral administration of phycocyanin and phycocyanobilin may offer a novel and feasible therapeutic approach for preventing diabetic nephropathy.

Phycocyanin, which is derived from *Spirulina platensis* a blue-green algae belonging to the cyanobacteria family, is a natural dye in food and cosmetics and is also used for the production of pharmaceuticals. Therefore, we hypothesized that bilirubin and phycocyanobilin may play an important role in mediating the vascular complications of diabetes, including nephropathy (1, 10, 17, 25). How- ever, very few trials have shown the effectiveness of antioxidant mechanisms is urgently needed. Oxidant stress is thought to play an important role in mediating the vascular complications of diabetes, including nephropathy (1, 10, 17, 25). However, very few trials have shown the effectiveness of antioxidants in humans. In recent years, bilirubin has been recognized as a powerful endogenous antioxidant in humans (30). Indeed, accumulating evidence has demonstrated an inverse relationship between bilirubin levels and cardiovascular diseases in humans, suggesting a beneficial effect of bilirubin on cardiovascular disease (4, 12, 21, 26, 28). We recently reported that diabetic patients with mild lifelong hyperbilirubinemia caused by Gilbert syndrome had a remarkably lower prevalence of diabetic nephropathy, retinopathy, and ischemic heart disease, along with reduced levels of markers of oxidative stress and inflammation compared with other diabetic patients (15).

**DIABETIC NEPHROPATHY** is a leading cause of end-stage renal failure. Therefore, a therapeutic approach targeting its causative mechanisms is urgently needed. Oxidative stress is thought to play an important role in mediating the vascular complications of diabetes, including nephropathy (1, 10, 17, 25). However, very few trials have shown the effectiveness of antioxidants in humans. In recent years, bilirubin has been recognized as a powerful endogenous antioxidant in humans (30). Indeed, accumulating evidence has demonstrated an inverse relationship between bilirubin levels and cardiovascular diseases in humans, suggesting a beneficial effect of bilirubin on cardiovascular disease (4, 12, 21, 26, 28). We recently reported that diabetic patients with mild lifelong hyperbilirubinemia caused by Gilbert syndrome had a remarkably lower prevalence of diabetic nephropathy, retinopathy, and ischemic heart disease, along with reduced levels of markers of oxidative stress and inflammation compared with other diabetic patients (15).

**MATERIALS AND METHODS**

**Animals.** Male C57BL/Ks J *db/db* mice and age- and sex-matched lean control *db/+* mice were purchased from Clea Japan (Tokyo, Japan). All mice were bred under pathogen-free conditions at Kyushu University Animal Center, Fukuoka, Japan. The animals had free access to tap water and standard chow (Clea Japan) containing 50.1% carbohydrates, 25.1% protein, 7.1% minerals, 4.5% fat, and 4.3% cellulose. At 12 wk of age, *db/db* mice and control *db/+* mice were randomly assigned to receive a powdered diet (Clea Japan) supplemented with or without phycocyanin (300 mg/kg) for 10 wk or phycocyanobilin (15 mg/kg) for 2 wk. *db/+* mice were given the same vehicle diet. Phycocyanin and phycocyanobilin (89% purity), extracted from phycocyanin, were kindly provided by Dainippon Ink and Chemicals, (Tokyo, Japan). Powdered diet was stored at −4°C in the dark. On the last day of phycocyanin or phycocyanobilin administration, all mice were killed by exsanguination under deep anesthesia by intraperitoneal injection of ketamine (40 mg/kg) and xylazine (20 mg/kg). Kidneys were immediately excised and frozen in liquid nitrogen and stored at −80°C or in formalin liquid for the following...
**Fig. 1.** Chemical structures of biliverdin, bilirubin, phycocyanin, and phycocyanobilin.

**Table 1.** Effects of phycocyanin on body weight and blood glucose

<table>
<thead>
<tr>
<th></th>
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<th>db/+ + PC</th>
<th>db/db</th>
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<td>Body weight, g</td>
<td></td>
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<tr>
<td>Baseline</td>
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<td>28.6 ± 0.3</td>
<td>53.4 ± 0.7*</td>
<td>52.5 ± 0.4†</td>
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<tr>
<td>5 wk</td>
<td>30.8 ± 0.4</td>
<td>29.7 ± 0.3</td>
<td>54.6 ± 0.6*</td>
<td>53.1 ± 1.6†</td>
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<tr>
<td>10 wk</td>
<td>33.1 ± 0.4</td>
<td>31.7 ± 0.5</td>
<td>57.1 ± 0.8*</td>
<td>55.2 ± 2.5‡</td>
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<td>FBS, mg/dl</td>
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<tr>
<td>Baseline</td>
<td>110.3 ± 3.1</td>
<td>107.8 ± 5.3</td>
<td>514.5 ± 8.2*</td>
<td>519.0 ± 13.1†</td>
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<tr>
<td>5 wk</td>
<td>108.8 ± 5.3</td>
<td>103.0 ± 4.6</td>
<td>534.3 ± 25.1*</td>
<td>509.0 ± 26.5†</td>
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<tr>
<td>10 wk</td>
<td>105.5 ± 3.8</td>
<td>107.3 ± 4.1</td>
<td>526.3 ± 26.4*</td>
<td>494.9 ± 25.2‡</td>
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Values are expressed as means ± SE; n = 8 mice per group. FBS: fasting blood glucose; PC, phycocyanin. *P < 0.01 vs. db/+ mice; †P < 0.01 vs. PC-treated db/+ mice.
GTCTTG-3′ (antisense); HO-1, 5′-AGGTGATGCTGACAGAGGAA-CAC-3′ (sense) and 5′-GAGATAGCAAATCGGCTGACG-3′ (antisense); MCP-1, 5′-GCAGTAAACGCCCCACTCA-3′(sense) and 5′-CCAGCCTACTCCTATGCAAC-3′ (antisense); β-actin, 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ (sense) and 5′-ATGGAGCCACCGATCCACA-3′ (antisense).

β-actin was used as an internal control. The specificity of PCR amplification was confirmed by melting curve analysis and agarose gel electrophoresis.

Western blot analysis. To determine Nox4 protein expression in the kidneys, renal tissues were homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA) and centrifuged at 12,000 rpm for 5 min. The protein concentration was measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Protein samples (30 μg per lane) were separated by 4–15% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories), and incubated with anti-Nox4 (1:1,000; Abcam, Cambridge, MA) and anti-β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO) primary antibodies. The membranes were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (1:5,000; Santa Cruz Biotech-

Fig. 2. Effects of oral administration of phyccyanin and phycocyanobilin on urinary 8-OHdG and 8-epi-PGF2α excretion. Urinary 8-OHdG excretion (A) and 8-epi-PGF2α (B) levels after 5 and 10 wk of treatment with phycocyanin. C: urinary 8-OHdG excretion and 8-epi-PGF2α excretion levels after 2 wk of treatment with phycocyanobilin. Data are shown as means ± SE (n = 8 per group). n.s., not significant. †P < 0.05, ‡P < 0.01.
nology) or sheep anti-mouse IgG antibody (1:5,000; GE Healthcare UK, Buckinghamshire, UK) for 1 h at room temperature. Images were acquired using an ECL-plus system (GE Healthcare).

**DHE staining.** Dihydroethidium (DHE) was used to determine in situ production of superoxide, as previously described (6). Mice were injected with 1 ml DHE (1 mg/ml in PBS; Invitrogen) into the right jugular vein under isoflurane anesthesia for 2 h. The mice were then killed by transcardial perfusion with 50 ml of 4% formaldehyde in PBS. The kidneys were frozen immediately in OCT compound (Tissue-Tech, Sakura Fine Chemical, Tokyo, Japan) and cut into 10-μm-thick sections on a cryostat. Nuclear staining was detected by Hoechst 33258 (Invitrogen) in PBS for 15 min in a dark chamber. Fluorescence images were obtained using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan). The fluorescence intensity was quantified using Adobe Photoshop software (version 6.0; Adobe Systems, Mountain View, CA). Each glomerulus was selected manually in ×400 images, and the mean value of the histogram on the red-colored channel was determined as the fluorescent level of DHE

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**Fig. 3.** Effects of oral administration of phyco- cyanin and phycocyanobilin on oxidative stress markers in renal tissues. Representative micrographs showing renal 8-OHdG staining after phycocyanin treatment (A) and semiquantitative analysis (B). Representative micrographs showing renal 8-OHdG staining after phycocyanobilin treatment (C) and semiquantitative analysis (D). Representative micrographs showing dihydroethidium (DHE) staining after phycocyanobilin treatment (E) and semiquantitative analysis (F). Values are expressed as means ± SE; n = 8 mice per group. n.s., not significant. †P < 0.05, ‡P < 0.01.
Fig. 4. Effects of oral administration of phycocyanin and phycocyanobilin on renal expression of NAD(P)H oxidase subunits. A: relative mRNA expression of NAD(P)H oxidase subunits Nox4, p22phox, and p47phox. B: representative micrographs showing renal Nox4 staining. C: representative Western blots showing renal Nox4 and β-actin protein expression. D: quantitative analysis of Nox4 protein expression after phycocyanin treatment. E: relative mRNA expression of NAD(P)H oxidase subunits. F: representative Western blots showing renal Nox4. G: quantitative analysis of Nox4 protein expression after phycocyanobilin treatment. Nox4 protein expression was normalized to the level of β-actin, and values are mean percentages of db/+ mice ± SE (n = 8 mice per group). n.s., not significant. †P < 0.05, ‡P < 0.01.
in each glomerulus. The mean glomerular fluorescent levels in each image were compared among the groups.

**Morphologic study.** To assess the extent of glomerular injury, renal cross sections were fixed in 10% formaldehyde and embedded in paraffin. The paraffin-embedded sections were cut into 3-μm-thick sections and stained with Periodic acid Schiff (PAS). Mesangial expansion and enlargement of the glomeruli were evaluated on the basis of the PAS-positive area and the total glomeruli area using Adobe Photoshop software and Scion imaging software (Scion, Frederick, MD).

**In vitro assay.** Normal human mesangial cells (NHMCs) were purchased from Lonza (Walkersville, MD). Mesangial cells were cultured in mesangial cell growth medium (Lonza) containing 5% FCS. Cells from passages 2–4 were used in the experiments. The

![Fig. 5](http://ajpregu.physiology.org/)

Fig. 5. Effects of oral administration of phycocyanin and phycocyanobilin on renal expression of mRNA for inflammatory makers and antioxidative enzyme hemeoxygenase-1. TNF-α, MCP-1, and hemeoxygenase-1 mRNA expression measured by real-time RT-PCR after phycocyanin treatment (A) and after phycocyanobilin treatment (B). The mRNA levels were normalized to the level of β-actin, and values are expressed as the mean percentage of levels in untreated db/+ mice ± SE (n = 8 mice per group), n.s., not significant. †P < 0.05, ‡P < 0.01.

![Fig. 6](http://ajpregu.physiology.org/)

Fig. 6. Effects of oral administration of phycocyanin and phycocyanobilin on urinary albumin excretion. Urinary albumin excretion (μg/day) after 5 and 10 wk phycocyanin treatment (A) and urinary albumin excretion (μg/day) after 2 wk of phycocyanobilin treatment (B). Values are expressed as means ± SE; n = 8 mice per group, n.s.; not significant. †P < 0.05, ‡P < 0.01.
cellular production of superoxide anions was determined by a lucigenin-
enhanced chemiluminescence assay, as previously described, with
minor modifications (6, 22). For the experiments, after NHMCs were
incubated with or without different concentrations of bilirubin, biliv-
eraldin, and phycocyanobilin at 0.3–2 μM for 24 h, they were detached
with trypsin/EDTA and resuspended in modified HEPES buffer con-
taining (in mM) 140 NaCl, 5 KCl, 0.8 MgCl2, 1.8 CaCl2, 1.0
Na2HPO4, 25 HEPES, and 1% glucose (pH 7.2). The cell suspension
was gently agitated with 0.1% Triton-X100 for cell permeabilization.
After preincubation with dark-adapted lucigenin (50 μM) for 10 min
at 37°C, NADPH (100 μM) was added to the cells immediately before
recording. Light emission was recorded every 10 s for 10 min and was
expressed as relative light units. NHMCs were preincubated with 10
μM diphenylene iodonium chloride (DPI), an NADPH oxidase inhib-
itor, for 1 h, to confirm the experimental specificity for NADPH
oxidase activity. Experiments were performed in triplicate, and all
results are from four independent experiments. After mixing the cell
suspension well, protein content was measured by BCA protein assay
reagent kit (Pierce). Superoxide production was calculated as the sum
of the relative light units per microgram protein. The effect of
phycocyanobilin on intracellular production of superoxide was also
evaluated in cultured NHMCs using DHE staining as described above.
The cells were plated in a glass-bottom dish (MatTek Co, Ashland,
MA). To examine the effect of phycocyanobilin on high glucose
level-induced superoxide production, the cells were incubated with
5.5 mM or 25 mM glucose for 3 days. Phycocyanobilin (1 μM) was
added concomitantly for the last 24 h of the incubation. Then, the cells
were replaced with the PBS containing DHE 20 μmol/l (Invitrogen,
Carlsbad, CA), and after 5 min incubation, nuclear staining was
performed using Hoechst 33258 (Invitrogen) for 10 min in a dark
chamber and rinsed with distilled H2O. Fluorescence images were
then obtained using a fluorescence microscope (model BZ-9000;

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**Fig. 7.** Effects of oral administration of phy-
cocyanin and phycocyanobilin on renal mesan-
gial expansion and renal expression of mRNA
for TGF-β and fibronectin. A: representative
micrographs showing renal sections stained
with Periodic Acid Schiff. B: semiquantitative
analysis of mesangial area. Results are ex-
pressed as mean percentage of the area in
untreated db/+ mice. TGF-β and fibronec-
tin mRNA expression measured by real-
time reverse-transcriptase PCR after phy-
cocyanin treatment (C) and after phycocya-
nobilin treatment (D). The mRNA levels were
normalized to the level of β-actin, and values
are expressed as the mean percentage of levels
in untreated db/+ mice ± SE (n = 8 mice per
group). n.s., not significant. †P < 0.05, ‡P <
0.01.
Keyence, Osaka, Japan). As for ANG II stimulation, after pretreatment of the cells with phycocyanobilin for 24 h, ANG II (1 μM) was added to nontreated cell and phycocyanobilin-pretreated cells for 2 h. Then, the cells were replaced with the PBS containing DHE 20 μmol/l, and then fluorescence images were obtained as above. DPI was concomitantly added for the last 1 h of high glucose incubation and ANG II incubation as a positive control of NAD(P)H oxidase inhibition.

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed using one-way ANOVA with Fisher’s protected least significant difference test. Values of P < 0.05 were considered statistically significant.

RESULTS

Effects of oral phycocyanin treatment on oxidative stress and renal function in db/db mice. Oral administration of phycocyanin (300 mg/kg daily) for 10 wk did not significantly affect body weight or blood glucose levels (Table 1). The urinary 8-OHdG and 8-epi-PGF_{2α} levels, markers of superoxide production, were significantly higher in db/db mice than in control db/+ mice. Phycocyanin treatment completely normalized the levels of these markers in db/db mice to control levels at 10 wk (Fig. 2, A and B). Immunostaining analysis showed that staining intensity of 8-OHdG was significantly increased in the renal tissues of diabetic mice at 10 wk, and these increases were normalized by phycocyanin treatment (Fig. 3, A and B). In parallel with the accumulation of oxidative stress markers, the mRNA levels of Nox4, a major subunit of NAD(P)H oxidase in the kidney, and the other renal NAD(P)H oxidase subunits were significantly increased in diabetic kidneys, and these increases were normalized by phycocyanin (Fig. 4A). The increased levels of Nox4 protein in diabetic kidneys were also normalized by phycocyanin treatment, as evaluated by immunostaining (Fig. 4B) and Western blot analysis (Fig. 4, C and D). The mRNA levels for inflammatory markers, TNF-α and MCP-1, and antioxidative enzyme hemooxygenase-1 were significantly increased in diabetic kidneys, and these were also normalized by phycocyanin treatment (Fig. 5A). To evaluate the therapeutic effects of phycocyanin, we examined its effects on albuminuria and renal mesangial expansion, one of the most striking histological characteristics of diabetic nephropathy in db/db mice. The administration of phycocyanin for 10 wk partially normalized albuminuria (Fig. 6A) and completely normalized mesangial expansion in the diabetic kidneys (Fig. 7, A and B). This effect was accompanied by normalized expression of tumor growth factor (TGF)-β, a key cytokine, which mediates extracellular matrix accumulation and glomerular expansion in diabetic kidneys and fibronectin, a predominant matrix protein (Fig. 7C).

Effects of oral phycocyanobilin treatment on oxidative stress. Phycocyanin contains an open-chain tetrapyrrole chromophore known as phycocyanobilin, which is covalently attached to the apoprotein. Next, we investigated the short-term effect of oral administration of phycocyanobilin extracted from phycocyanin (89% purity) on oxidative stress in diabetic kidneys. Phycocyanobilin treatment (15 mg/kg) for 2 wk did not significantly affect body weight, but it did significantly decrease blood glucose levels, although the magnitude of effect was small (Table 2). Similar to phycocyanin, phycocyanobilin normalized the increases in urinary 8-OHdG and 8-epi-PGF_{2α} levels (Fig. 2C), renal oxidative stress markers evaluated by renal 8-OHdG staining (Fig. 3, C and D) and DHE staining (Fig. 4, E and F), Nox4 mRNA (Fig. 4E), and protein expression (Fig. 4, F and G), as well as the mRNA levels of other NAD(P)H oxidase components (Fig. 4E), inflammatory markers and HO-1 (Fig. 5B). In addition, phycocyanin normalized albuminuria (Fig. 6B) and decreased the expression of TGF-β and fibronectin in diabetic kidneys (Fig. 7D).

In vitro effects of phycocyanobilin. The in vitro effects of bilirubin, biliverdin, and phycocyanobilin on NAD(P)H oxidase activities were evaluated by the lucigenin method in cultured NHMCs. Preincubating the cells with phycocyanobilin, biliverdin, or bilirubin for 24 h at concentrations from 300 nM to 20 μM dose dependently reduced NAD(P)H-dependent superoxide production (Fig. 8A). We also determined the effects of phycocyanobilin on intracellular oxidative stress by DHE staining. Preincubating the cells with 1 μM phycocyanobilin significantly attenuated high glucose-induced intracellular oxidative stress (Fig. 8B) and ANG II-induced intracellular oxidative stress (Fig. 8D).

DISCUSSION

We previously reported that the prevalence of vascular complications, including nephropathy, was reduced in diabetic patients with Gilbert syndrome, a congenital cause of hyperbilirubinemia, and reduced markers of oxidative stress (15). Meanwhile, Fukui et al. (7) reported that low-serum bilirubin levels were correlated with microalbuminuria and subclinical atherosclerosis in patients with Type 2 diabetes. In this context, we demonstrated that the administration of biliverdin, a precursor of bilirubin that is much more water soluble than bilirubin, inhibits albuminuria and renal histological abnormalities in db/db mice by inhibiting oxidative stress (6). However, for clinical use, supplemental daily intake of fairly large amounts of biliverdin might be required to achieve a meaningful impact on serum and tissue bilirubin levels, because endogenous production of heme has been estimated at 300–400 mg daily, giving rise to a nearly equivalent amount of bilirubin (23). In addition, chemical synthesis of biliverdin may be too complex and costly for human use. An alternative strategy might be the use of phycobilins, structural analogs of biliverdin synthesized by plants, algae, and cyanobacteria, because it may be feasible to produce commercial quantities of phycobilin. In addition, it has been already confirmed that phycobilins are good substrates for the ubiquitously expressed enzyme biliverdin reductase with K_m values similar to those of biliverdin.

<table>
<thead>
<tr>
<th>Body weight, g</th>
<th>db/+</th>
<th>db++ PCB</th>
<th>db/db</th>
<th>db/db+ PCB</th>
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<tr>
<td>Baseline</td>
<td>26.8 ± 0.3</td>
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<td>49.1 ± 0.2*</td>
<td>49.2 ± 0.2†</td>
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<td>2 wk</td>
<td>28.3 ± 0.4</td>
<td>28.7 ± 0.6</td>
<td>51.0 ± 0.4*</td>
<td>50.1 ± 0.4†</td>
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<tr>
<td>FBS, mg/dl</td>
<td>108.0 ± 2.9</td>
<td>110.0 ± 4.0</td>
<td>469.9 ± 15.1*</td>
<td>475.0 ± 12.4†</td>
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</table>

Values are expressed as means ± SE (n = 8 mice per group). FBS: fasting blood glucose; PCB, phycocyanobilin. *P < 0.01 vs. db/+ mice; †P < 0.01 vs. PCB-treated db/+ mice.
Fig. 8. Effects of phycocyanobilin on superoxide production in cultured human mesangial cells. A: dose-dependent inhibition of NAD(P)H-dependent superoxide production by bilirubin, biliverdin, and phycocyanobilin, evaluated by the lucigenin method, as described in detail in MATERIALS AND METHODS. Representative micrographs showing high glucose-induced superoxide production evaluated by dihydroethidium (DHE) staining (B) and semiquantitative analysis (C). Briefly, the cells were incubated with 5.5 mM (Normal) or 25 mM glucose (High) for 3 days, and phycocyanobilin was added concomitantly for the last 24 h of the incubation (High + PCB). D: representative micrographs showing ANG II-induced superoxide production, evaluated by DHE staining, as described in detail in MATERIALS AND METHODS. After pretreatment of the cells with phycocyanobilin for 24 h, ANG II (AT2) was added to nontreated cells (control) and phycocyanin-treated cells (AT2 + PCB). Diphenylene iodonium chloride (DPI) was concomitantly added for the last 1 h of high-glucose incubation and ANG II incubation as a positive control of NAD(P)H oxidase inhibition. The relative intensity of DHE staining was compared with nontreated control. E: semiquantitative analysis. Values are percentages of nontreated control ± SE. n.s., not significant. †P < 0.05, ‡P < 0.01.
giving rise to phycorubins that are structural analogs of bilirubin (31). Phycobilins are generally ligated to apoproteins to generate protein-chromophore complexes known as phycocyanins. Thus, phycobilins and phycocyanins are expected to have antioxidant activity (2, 3, 27). In this study, we used phycocyanin and phycocyanobilin from spirulina, because spirulina and phycocyanin are widely used as nutrients for human and animal consumption, as natural dyes in food and cosmetics, and are also used for the production of pharmaceuticals. We showed that oral administration of phycocyanin (300 mg/kg daily) for 10 wk protected against albuminuria and renal mesangial expansion and normalized the expression of TGF-β and fibronectin in db/db mice by inhibiting renal oxidative stress. Treatment for 2 wk with phycocyanobilin (15 mg/kg) obtained from phycocyanin (89% purity) also normalized urinary and renal oxidative stress markers, as well as albuminuria in db/db mice. These data suggested that the effect of phycocyanin may be mediated by that of phycocyanobilin. Although the antioxidant effects of bilirubin are due to its strong radical scavenging activity, several reports have shown that bilirubin directly inhibits NAD(P)H oxidase activity (19, 20). Interestingly, we and other investigators have shown that nonphagocytic NAD(P)H oxidases may be the main sources of ROS in the vascular tissues of diabetic animals and patients (11, 13, 16, 18). Therefore, inhibiting vascular NAD(P)H oxidase might offer a potent therapeutic approach to prevent diabetic vascular complications, including nephropathy (14). In this study, we showed that phycocyanobilin, similar to bilirubin and biliverdin, inhibited NADPH-induced superoxide production in cultured mesangial cells, suggesting that phycocyanobilin was capable of inhibiting NAD(P)H oxidase activity. In addition, phycocyanobilin inhibited high glucose-induced and ANG II-induced superoxide production derived from NAD(P)H oxidase, although neither phycocyanobilin nor NADPH oxidase inhibitor seemed to have any effect on baseline superoxide production measured by DHE staining in cells of the very same cell line, which was probably derived from other sources. We also showed that oral administration of phycocyanin and phycocyanobilin prevented increases in the expression of the NAD(P)H oxidase component Nox4, which is thought to be the major source of oxidative stress in diabetic kidneys (5, 8, 29). Gorin et al. (9) revealed that downregulating Nox4 by antisense oligonucleotides completely suppressed oxidative stress and thus prevented renal hypertrophy and fibronectin expression in diabetic rats. Therefore, normalization of Nox4 expression by phycocyanin and phycocyanobilin could play an important role in reducing oxidative stress and preventing renal dysfunction in the diabetic kidney. In terms of the relationship between the activity and expression of NAD(P)H oxidase, it should be noted that the rapid activation of NAD(P)H oxidase may induce the expression of NAD(P)H oxidase components, and, hence, further enhance ROS generation. One report showed that activation of NAD(P)H oxidase induces the expression of Ets-1, a downstream transcriptional effector of ROS, by a redox-sensitive mechanism, which then induces the expression of NAD(P)H oxidase components, such as p47phox (24). Therefore, the normalization of Nox4 expression may be at least partly explained by both the radical scavenging activity and the inhibitory effects on NAD(P)H oxidase activity of phycocyanin and phycocyanobilin. However, it is also possible that these compounds directly affect the expression of NAD(P)H oxidase components, including Nox4. The detailed molecular mechanism should be evaluated in future studies.

In conclusion, we showed for the first time that oral administration of phycocyanin and phycocyanobilin extracted from Spirulina platensis protects against renal dysfunction in db/db mice by inhibiting oxidative stress.

**Perspectives and Significance**

Because spirulina is used as a nutritional supplement in many countries, it may be feasible to produce commercial quantities of phycocyanin. This study suggests that oral administration of phycocyanin and phycocyanobilin may represent a feasible and novel therapeutic approach to prevent diabetic nephropathy. Future studies will incorporate results from clinical trials.

**ACKNOWLEDGMENTS**

The authors thank Daiinippon Ink & Chemicals, (Tokyo, Japan) for kindly providing phycocyanin and phycocyanobilin extracted from phycocyanin. We also appreciate the technical support provided by the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

**DISCLOSURES**

Toyoshi Inoguchi and Mark F. McCarty are coinventors of a pending patent on phycocyanobilin oligopeptides as NAD(P)H oxidase inhibitors.

**AUTHOR CONTRIBUTIONS**


**GRANTS**

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**REFERENCES**


