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

**Jing Zheng,**1 Toyoshi Inoguchi,1,2 Shuji Sasaki,1 Yasutaka Maeda,1 Mark F. McCarty,3 Masakazu Fuji1, Noriko Ikeda,1 Kunihisa Kobayashi,1 Noriyuki Sonoda,1,2 and Ryoichi Takayanagi1

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.

These findings raise the hypothesis that bilirubin may inhibit the development of diabetic complications, including nephropathy. In this context, we previously reported that biliverdin, a precursor of bilirubin, inhibited the development of renal abnormalities in *db/db* mouse, a rodent model of Type 2 diabetes, by inhibiting oxidative stress (6). Taken together, these findings indicate that increasing bilirubin levels may represent a novel therapeutic approach to prevent diabetic nephropathy.

Phycocyanin is derived from *Spirulina platensis* a blue-green algae belonging to the cyanobacteria family. It contains an open-chain tetrapyrrole chromophore known as phycocyanobilin, which is covalently attached to the apoprotein. Phycocyanobilin has a chemical structure similar to that of biliverdin and seems to be susceptible to biliverdin reductase, giving rise to phycocyanorubin, an analog of bilirubin (31). The chemical structure of phycocyanobilin and phycocyanin is shown in Fig. 1. Therefore, phycocyanobilin and phycocyanin are expected to have antioxidant effects similar to biliverdin and bilirubin. Indeed, several reports have shown that phycocyanin has antioxidant, anti-inflammatory, and radical scavenging properties (2, 3, 27). Phycocyanin is also widely used as a natural dye in food and cosmetics and is also used for the production of pharmaceuticals. Therefore, we hypothesized that the administration of phycocyanin or phycocyanobilin could represent a feasible therapeutic approach to prevent diabetic nephropathy. In this study, we investigated the effects of orally administered phycocyanin and phycocyanobilin on oxidative stress and renal abnormalities in *db/db* mice.

**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
experiments. All protocols were reviewed and approved by the Ethics Committee of Animal Experiments, Graduate School of Medical Sciences, Kyushu University.

Urine analysis. Twenty-four-hour urine collection was performed using a metabolic cage on the last day of treatment. To evaluate oxidative stress, we measured urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$) levels using competitive enzyme-linked immunosorbent assay kits (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan) containing phenol and guanidine isothiocyanate. RNA concentrations were determined spectrophotometrically. RNA was purified using RNeasy Mini Kits (Qiagen, Gaithersburg, MD) and reverse transcribed using QuantiTect Reverse transcription kit (Qiagen) for DNA synthesis. Quantification of mRNA expression levels was performed on a Chromo4 real-time PCR Detector with IQ SYBR Green Supermix reagent (Bio-Rad Laboratories, Hercules, CA). PCR reactions for each target cDNA were done using the following primer pairs: Nox4, 5′-ATTTGGGATAGGCTCCAG-GCAAAC-3′ (sense) and 5′-CACATGGGTATAAGCTTTGT-TGGCTACTGCTGGACG-3′ (antisense); P47phox, 5′-CTCCAGCAGACAGATGAGCACAC-3′ (sense) and 5′-TTTCAC-3′ (antisense); P22phox, 5′-GCAAAC-3′ (sense) and 5′-CTTGCTCAGCCTGCCTTCTTAG-3′ (antisense); TGF-β, 5′-GCAATGGGCTGAACCAAGGA-3′ (sense) and 5′-GACGT-GAGCCGCTGAATCCGA-3′ (antisense); fibrocin, 5′-ACATGGCTTATTGGGACAAAC-3′ (sense) and 5′-ACATCGGCGAGTATCG-3′ (antisense).

RNA extraction and real-time PCR. The excised organs were immediately stored in RNAlater solution (Ambion, Austin, TX) at −80°C for semiquantitative RT-PCR. Total RNA was extracted from frozen tissue samples using ISOGEN (Nippon Gene, Tokyo, Japan) containing phenol and guanidine isothiocyanate. RNA concentrations were determined spectrophotometrically. RNA was purified using RNeasy Mini Kits (Qiagen, Gaithersburg, MD) and reverse transcribed using QuantiTect Reverse transcription kit (Qiagen) for DNA synthesis. Quantification of mRNA expression levels was performed on a Chromo4 real-time PCR Detector with IQ SYBR Green Supermix reagent (Bio-Rad Laboratories, Hercules, CA). PCR reactions for each target cDNA were done using the following primer pairs: Nox4, 5′-ATTTGGGATAGGCTCCAG-GCAAAC-3′ (sense) and 5′-CACATGGGTATAAGCTTTGT-TGGCTACTGCTGGACG-3′ (antisense); P47phox, 5′-CTCCAGCAGACAGATGAGCACAC-3′ (sense) and 5′-TTTCAC-3′ (antisense); P22phox, 5′-GCAAAC-3′ (sense) and 5′-CTTGCTCAGCCTGCCTTCTTAG-3′ (antisense); TGF-β, 5′-GCAATGGGCTGAACCAAGGA-3′ (sense) and 5′-GACGT-GAGCCGCTGAATCCGA-3′ (antisense); fibrocin, 5′-ACATGGCTTATTGGGACAAAC-3′ (sense) and 5′-ACATCGGCGAGTATCG-3′ (antisense).

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

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<tr>
<th>Time</th>
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<th>db/db</th>
<th>db/db + PC</th>
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<tr>
<td>Baseline</td>
<td>29.3 ± 0.4</td>
<td>28.6 ± 0.3</td>
<td>53.4 ± 0.7*</td>
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<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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<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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<tr>
<td>FBS, mg/dl</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.

Fig. 1. Chemical structures of biliverdin, bilirubin, phycocyanin, and phycocyanobilin.
GTCTTG-3' (antisense); HO-1, 5'-AGGTGATGCTGACAGAGGAA-CAC-3' (sense) and 5'-GAGATAGCACAACGGCTGCAGCAG-3' (antisense); MCP-1, 5’-GCAGTTAAGCCACACTCA-3’ (sense) and 5’-CCAGCCTACTCATTCTATGCAAC-3’ (antisense); β-actin, 5’-CATCGTAAAGACTGGCTGACG-3' (antisense); and 5’-ATGGAGCCACCGATCCAACA-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-
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 33342 (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.

Fig. 5. Effects of oral administration of phycocyanin and phycocyanobilin on renal expression of mRNA for inflammatory markers and antioxidative enzyme hemoxygenase-1. TNF-α, MCP-1, and hemoxygenase-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.

A

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Urinary albumin excretion

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, biliverdin, and phycocyanobilin at 0.3–2 μM for 24 h, they were detached with trypsin/EDTA and resuspended in modified HEPES buffer containing (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 inhibitor, 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;
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). Next, we measured the expression of NAD(P)H oxidase, a major source of reactive oxygen species (ROS) in the kidney. 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, the mRNA levels of Nox4, a major subunit of NAD(P)H oxidase (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, bilirubin, or biliverdin for 24 h at concentrations from 300 to 2 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 Km values similar to those of biliverdin.
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.
compounds directly affect the expression of NAD(P)H oxidase anin and phycocyanobilin. However, it is also possible that these inhibitory effects on NAD(P)H oxidase activity of 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 (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 phycobilin. 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**

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**GRANTS**

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


