Eur J Nutr (2013) 52:601–608
DOI 10.1007/s00394-012-0362-0

ORIGINAL CONTRIBUTION

Chlorella pyrenoidosa ameliorated L-NAME-induced hypertension and cardiorenal remodeling in rats

Su-Ching Yang · Hsin-Yi Yang · Yi-Ching Yang · Hsiang-Chi Peng · Pei-Yin Ho

Received: 8 August 2011 / Accepted: 16 April 2012 / Published online: 3 May 2012

Abstract
Purpose Hypertension is one of the main factors causing cardiovascular diseases. The aim of the study is to investigate the effects of *Chlorella pyrenoidosa* on blood pressure and cardiorenal remodeling in rats with N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced endothelial dysfunction.
Methods Rats were fed a diet containing L-NAME (40 mg/kg) with or without chlorella (4 or 8 %) for 5 weeks. We found that chlorella retarded the development of hypertension and cardiorenal remodeling during the 5-week experimental period.
Results Although there was no difference in NO_x levels or plasma arginine concentrations, plasma and tissues ACE activities were significantly lower in the chlorella groups than in the L-NAME group. Moreover, tissue tumor necrosis factor-α concentrations and renal CYP4A expression were also lower in the chlorella group.
Conclusion These results suggest that chlorella might ameliorate the elevation of blood pressure and show cardiorenal-protective effects in nitric oxide-deficient rats, and one possible mechanism might be mediated by its ACE inhibitory activity.

Keywords Chlorella · Hypertension · Nitric oxide · Angiotensin-converting enzyme

S.-C. Yang · Y.-C. Yang · H.-C. Peng
School of Nutrition and Health Sciences, Taipei Medical University, Taipei, Taiwan

H.-Y. Yang (✉)
Department of Nutrition, I-Shou University, No. 8, Yida Rd., Jiaosu Village, Yanchao District, Kaohsiung City 82445, Taiwan
e-mail: aliceyang@isu.edu.tw

Y.-C. Yang
Department of Nutrition, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

P.-Y. Ho
Graduate Institute of Oncology, National Taiwan University, Taipei, Taiwan

Introduction

Hundreds of millions of people suffer from hypertension. According to the World Health Report 2002, seven million people died of hypertension worldwide. Complications of hypertension, such as coronary heart disease, stroke, arrhythmia, heart failure and abnormal renal dynamics, may damage the cardiovascular system.

Endothelial dysfunction can be observed in early stages of hypertension [1]. Endothelial function is modulated by vasodilators and vasoconstrictors in vivo. The imbalance between the main vasodilator (nitric oxide, NO) and vasoconstrictor (angiotensin II) impairs endothelial function. Treating rats with N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) causes injuries to the vascular endothelium, and this model is widely used to the study of hypertension, and cardiovascular and kidney diseases [2]. Endothelial dysfunction exhibits a reduction in NO bioavailability and also results in cardiovascular remodeling through the activation of the renin-angiotensin system (RAS) to produce angiotensin II. Therapy of hypertension with angiotensin-converting enzyme (ACE) inhibitors may inhibit the formation of angiotensin II and thus, suppress vasoconstriction, inflammation, and oxidative stress and lower blood pressure [3].

Recently, chlorella was reported to be beneficial to health because it is rich in plant proteins and other nutrients

such as ascorbic acid, tocopherol and L-arginine. Previous studies indicated that *Chlorella vulgaris* may have antioxidative and antihypertensive effects [4]. In a study of SHR-SP, 20 % of chlorella in the diet was needed to lower blood pressure and increase survival rates of rats [5]. However, daily supplementation with 10 g *Chlorella pyrenoidosa* for 2 months in mildly to moderately hypertensive subjects did not affect the systolic blood pressure [6]. Reports about the effects of chlorella on hypertension are very few and still remain inconsistent. Therefore, the aim of the present study was to investigate the effects of different dosages of chlorella supplementation on blood pressure and cardiorenal remodeling in an endothelial dysfunction model.

Materials and methods

Experiment design

Forty male Wistar rats (8 weeks old) were purchased from the Laboratory Animal Center of National Taiwan University (Taipei, Taiwan). Rats were housed in individual cages under a 12-h light–dark cycle at 23 ± 2 °C and a relative humidity of 55 ± 10 %. Rats were divided into four groups (10 rats for each group) and fed a diet based on AIN-93 M (Table 1) for 5 weeks containing different amounts of *C. pyrenoidosa* (Taiwan Chlorella Co., Taipei, Taiwan): a control group (C); an L-NAME group (L); rats fed 4 % chlorella (G1); and rats fed 8 % chlorella (G2). Rats in the L, G1 and G2 groups were orally given 40 mg/kg per day of L-NAME in drinking water (Fluka, Buchs, Switzerland) during the experiment period. Food and water were provided with free access. The body weight and food intake were recorded every week. This animal experiment was approved by the University Committee for Animal Care and Use and following the guidelines of the National Animal Research Center (Taipei, Taiwan).

Table 1 Diet composition (%)

Ingredients*	C	L	G1	G2
Cornstarch	46.57	46.57	45.97	45.37
Dextrin	15.5	15.5	15.5	15.5
Casein	14	14	11.6	9.2
Sucrose	10	10	10	10
Soy oil	4	4	3.68	3.36
Mineral mix	3.5	3.5	3.5	3.5
Cellulose	5	5	4.8	4.6
Vitamin mix	1	1	1	1
Cystine	0.18	0.18	0.18	0.18
Choline	0.25	0.25	0.25	0.25
Chlorella	–	–	4	8

* Cornstarch was from the Samyang Genex (Seoul, Korea). Dextrin, casein (high-N), cellulose (non-nutritive bulk), mineral mix (AIN-93 M), vitamin mix (AIN-93 M), cystine were from the ICN Biochemicals (Aurora, OH, USA). Choline and cholic acid were from the Acros (NJ, USA). Sucrose and soy oil were from the Taiwan Sugar Co. (Taipei, Taiwan). *Chlorella pyrenoidosa* was from the Taiwan Chlorella Co. (Taipei, Taiwan) containing 60 % protein, 15 % carbohydrate, 8 % lipids and 5 % fiber. C: control group, L: L-NAME group; G1: 4 % chlorella group; G2: 8 % chlorella group

Measurement of blood pressure

Blood pressure was measured at 0, 2 and 4 weeks intervals during the experimental period by the tail-cuff method with an electrosphygomanometer (MK-2000ST, Muromachi, Tokyo, Japan). Rats were put into restrainers and at least five readings were recorded. The maximum and minimum values were discarded, and the blood pressure was calculated as the average of the remaining three values.

Blood and tissue sampling

At the end of 5 weeks, rats were killed after being anesthetized with sodium pentobarbital. Blood samples were collected from the interior vena cava into tubes containing anticoagulant. Blood samples were immediately centrifuged, and the plasma was stored at −80 °C until being analyzed. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, uric acid, creatinine, urea nitrogen, sodium, potassium, calcium and phosphorus were determined using a Hitachi 7170 autoanalyzer (Tokyo, Japan). Hearts and kidneys were collected, weighed and divided into several parts for various analyses. Heart and kidney samples were homogenized in 400 mM phosphate buffer (pH 7.2) containing 340 mM sucrose, 900 mM NaCl and protease inhibitors. After centrifugation, the supernatants were stored at −80 °C. Urine samples (24 h) were collected using metabolic cages before killing the animals. Urinary protein and urea nitrogen excretion were also analyzed with an autoanalyzer. The creatinine clearance rate (CCr) was calculated as follows:

$$\begin{aligned} \text{CCr} = {} & \text{urine creatinine (mg/dL)} \\ & \times \text{ 24-h urine volume (mL)/plasma creatinine (mg/dL)} \\ & \times \text{ 1,440 (min)} \end{aligned}$$

Malonaldehyde (MDA) levels, tumor necrosis factor (TNF)-α, and plasminogen activator inhibitor (PAI)-1 concentrations

MDA in plasma was measured by the thiobarbituric acid-reactive substance method [7]. TNF-α and PAI-1 were measured with enzyme-linked immunosorbent assay

(ELISA) kits (rat TNF-α/TNFSF1A, R&D, USA; Zymutest rat-PAI-1 (antigen/activity), Hyphen BioMed, France).

Nitrite and nitrate (NO_x)

Due to the short half-life and low concentration of NO in vivo, we evaluated plasma and tissue NO levels by measuring its stable metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), by a modified Griess reaction method [8]. The principle of the assay is reduction of nitrate by vanadium (III) in combination with the detection by the acidic Griess reaction.

Free amino acid analysis

After a precolumn derivatization of the free amino acids, dabsyl derivatives were separated on a high-performance liquid chromatographic (HPLC) column (HYPERSIL Amino acid, Thermo, 4.5 × 150 mm). Fifty microliters of the derivatized standard or samples was injected. The mobile phase was composed of two eluents: buffer (A) 24 mM Na-acetate (pH 6.6): acetonitrate at 80:20 and buffer (B) acetonitrate: 2-propanol at 40:60. Elution was performed at a flow rate of 1 mL/min at 45 °C. Starting with 5 % B until 4 min and installing a gradient to obtain 20 % B at 8 min, 25 % B at 15 min, 60 % B at 27 min, and 100 % B at 28 min, and then maintaining 100 % B until 32 min. Detection was achieved with a ultraviolet–visible detector at 436 nm. Free amino acid quantification was accomplished by the absorbance recorded in the chromatograms relative to external standards (AAS18, Sigma, USA).

Plasma and tissue ACE activity

This assay was performed essentially according to a previously described spectrophotometric method performed in microtiter plate [9]. Samples or freshly prepared standard solutions were added into 1.75 mM furanacryloyl-prolyl-glycylglycine (FA-PGG) in 50 mM tris–HCl buffer (pH7.5). ACE activity was calculated as the rate of decrease in the absorbance at 340 nm during the first 20 min of catalyzed hydrolysis of FAPGG at 37 °C compared with standard solutions.

Pathohistological analysis

Dissected hearts and kidneys of the rats were fixed in 10 % formaldehyde solution. Samples were stained with hematoxylin and eosin (H&E). The biopsies were examined by a pathologist on a blinded basis. To evaluate inflammation, fibrosis and intimal thickening of the small arteries, the heart and kidneys were observed at 200× magnification.

Kidney cytochrome P450 4A expression

Kidneys were homogenized in 10 mmol/L potassium buffer with 250 mmol/L sucrose, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation, microsomal pellets were resuspended in 100 mmol/L potassium buffer (pH 7.3). Protein concentrations of the samples were measured with the Bradford method. Twenty micrograms of microsomal protein was separated by electrophoresis on 10 % sodium dodecylsulfate (SDS)-polyacrylamide gels for 2 h at 100 V. The proteins were transferred to a nitrocellulose membrane, and the membrane was blocked overnight in TBST-20 buffer containing 5 % nonfat dry milk at 4 °C. The membrane was incubated for 2 h with a polyclonal antibody against rat CYP4A (ABR, Golden, USA) at a 1:2,000 dilution in TBST buffer containing 1 % bovine serum albumin (BSA). The membrane was then washed with TBST buffer three times and incubated with goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Jackson, West Grove, USA) at 1:10,000 dilution in TBST buffer containing 1 % BSA. Blots were then washed several times with TBST and developed using an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Fisher's least significant difference test using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Results are expressed as the mean ± SEM. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Body weight, nutritional status, plasma and urine analyses, nitrite and nitrate concentrations

At the end of the study, we found no differences in body weight (387.7 ± 13.3 g, 386.2 ± 9.3 g, 390.3 ± 8.4 g, and 388.8 ± 8.4 g) or food intake among all groups, respectively.

In the analysis of plasma ALT, uric acid, creatinine, sodium, potassium and calcium concentrations, no differences were found among all groups. But we found that AST was significantly higher in the L group compared with the C group, and the concentration ion the G2 group was significantly lower than that of the L group. We also found that the G1 and G2 groups fed the diet containing 4 and 8 % chlorella had higher albumin concentrations than the L group (Table 2). In the plasma, the G2 group had a significantly lower PAI-1 level compared to the L group. We

Table 2 Body weight, food intake, plasma and urine analysis, and nitrite and nitrate concentrations in rats with L-NAME-induced hypertension

	C	L	G1	G2
Body weight (g)	388.7 ± 13.3	386.2 ± 9.3	390.3 ± 8.4	388.8 ± 8.4
Food intake (g/day)	23.1 ± 0.5	21.7 ± 0.6	21.9 ± 0.6	21.6 ± 0.5
Fluid intake (mL/day)	37.5 ± 1.3	37.4 ± 0.7	36.5 ± 1.0	35.7 ± 1.3
Plasma				
AST (IU/L)	68.4 ± 3.6^{b}	99.9 ± 11.6^{a}	87.2 ± 6.4^{ab}	77.6 ± 2.2^{b}
ALT (IU/L)	22.1 ± 0.8	22.6 ± 3.1	23.1 ± 1.0	22.1 ± 1.3
ALB (g/dL)	3.8 ± 0.0^{a}	3.6 ± 0.1^{b}	3.8 ± 0.1^{a}	3.8 ± 0.1^{a}
Uric acid (mg/dl)	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.3
Creatinine (mg/dL)	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
Urea nitrogen (mg/dL)	11.5 ± 0.2^{b}	14.5 ± 1.6^{ab}	16.9 ± 2.2^{a}	11.3 ± 0.7^{b}
Sodium (meq/L)	141.8 ± 0.4	140.9 ± 1.0	141.8 ± 0.8	141.5 ± 0.5
Potassium (meq/L)	6.3 ± 0.2	6.1 ± 0.2	6.0 ± 0.1	5.9 ± 0.3
Calcium (mg/dL)	10.5 ± 0.1	10.2 ± 0.1	10.5 ± 0.1	10.5 ± 0.1
Phosphorus (mg/dL)	6.1 ± 0.4^{b}	8.4 ± 0.4^{a}	7.9 ± 0.3^{a}	8.2 ± 0.2^{a}
PAI-1 (ng/mL)	0.8 ± 0.1^{b}	1.8 ± 0.4^{a}	1.1 ± 0.2^{ab}	0.9 ± 0.1^{b}
Urine				
Protein (mg/day)	4.6 ± 0.4^{b}	8.3 ± 0.4^{a}	7.0 ± 0.8^{ac}	4.8 ± 0.5^{bc}
Urea nitrogen (mg/day)	75.6 ± 4.4	84.7 ± 7.0	77.2 ± 3.3	71.6 ± 7.3
CCr (mL/min)	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
NO_x				
Plasma (μM/mL)	4.05 ± 0.52	3.92 ± 0.40	3.73 ± 0.35	3.94 ± 0.36
Aorta (mg protein)	38.39 ± 5.71^{a}	14.83 ± 1.65^{b}	17.65 ± 1.53^{b}	22.33 ± 5.60^{b}
Heart (mg protein)	1.85 ± 0.05	1.78 ± 0.06	1.76 ± 0.09	1.73 ± 0.05
Kidney (mg protein)	1.11 ± 0.14^{a}	0.74 ± 0.09^{b}	0.69 ± 0.07^{b}	0.68 ± 0.11^{b}

C, control group; L, L-NAME group; G1, rats fed 4 % chlorella; G2, rats fed 8 % chlorella. Values are presented as mean ± SEM. ($n = 8$). Different superscript letters in a row indicate a significant difference ($p < 0.05$)

found that the chlorella group showed no significant effects on MDA levels in the plasma, aorta, heart, or kidneys (data not shown).

In the urine analysis, urinary protein of the G2 group was significantly lower than that of the L-NAME group. There were also lower average values of urinary nitrogen concentrations in the chlorella groups, but no significant difference was found (Table 2).

In the free amino acid analysis, arginine levels in the plasma did not significantly differ among all groups. At the end of the study, the aorta and kidney NO_x levels were significantly lower in the L, G1 and G2 group than in the C group. NO_x levels in the plasma and heart did not significantly differ among all groups (Table 2).

Blood pressure

Rats treated with L-NAME for 2 weeks had significantly higher systolic (SBP) and diastolic blood pressure (DBP) compared to the baseline, and values continued to rise through the end of the study. The SBP and DBP of the G1 and G2 groups were significantly lower than those of the L group after 4 weeks. We also found that the SBP and DBP of the G2 group were significantly lower than those of the G1 group (Fig. 1a).

ACE activity

In Fig. 1b, plasma ACE activity of the L group was significantly higher than that of the C group from the second week. At the end of the study, ACE activities in the G1 and G2 groups were lower than that of the L group, and the effect was dose-dependent. ACE activity in the peripheral tissues was also important in cardiorenal remodeling and modulation of blood pressure. L-NAME led to elevation of ACE activity in the aorta, heart and kidneys. Rats consuming 4 and 8 % chlorella had significantly lower ACE activity in the aorta and kidneys (Fig. 2a).

TNF-α concentrations

Inhibition of NO synthesis by L-NAME significantly elevated TNF-α concentrations, a marker of inflammation, in the aorta, heart and kidneys. Chlorella supplementation decreased TNF-α concentrations in the aorta and heart and normalized TNF-α concentrations in the kidneys (Fig. 2b).

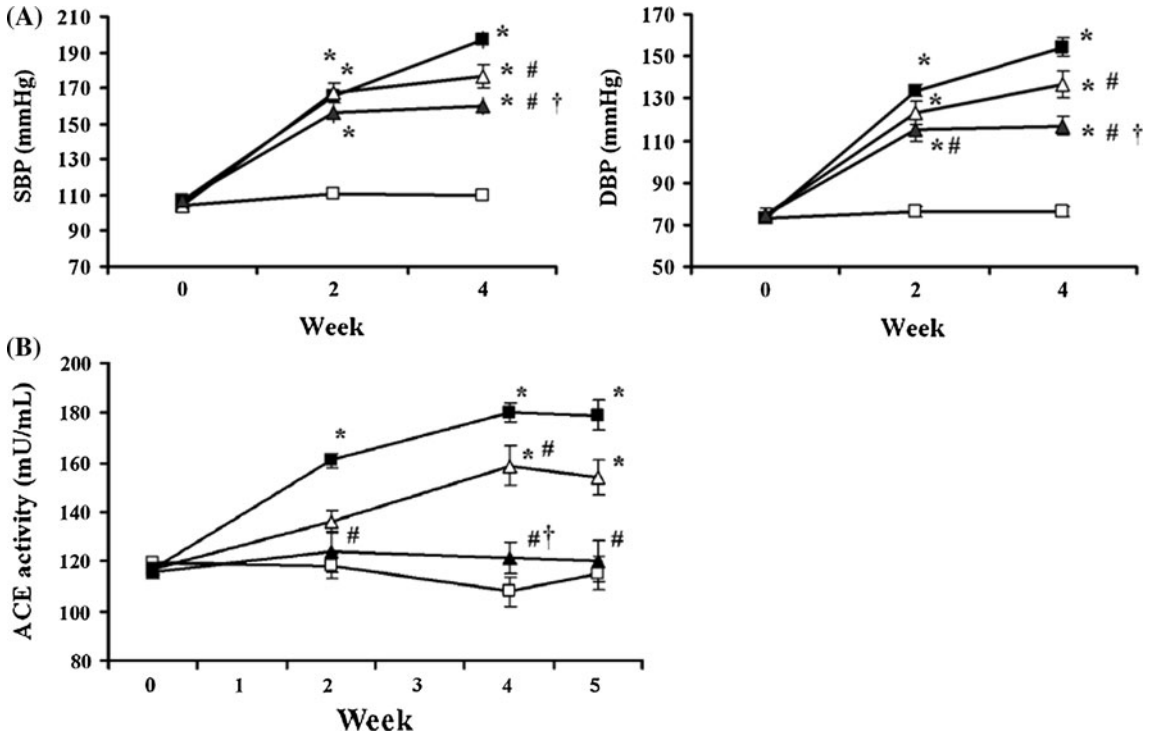

Fig. 1 Changes of blood pressure (**a**) and plasma ACE activity (**b**) in rats with L-NAME-induced hypertension. C, control group (*Open square*); L, L-NAME group (*filled square*); G1, rats fed 4 % chlorella (*Open triangle*); G2, rats fed 8 % chlorella (*filled triangle*). Values are presented as mean ± SEM. ($n = 8$). *Significantly different from the C group ($p < 0.05$) #significantly different from the L group ($p < 0.05$) †significantly different from the G1 group ($p < 0.05$)

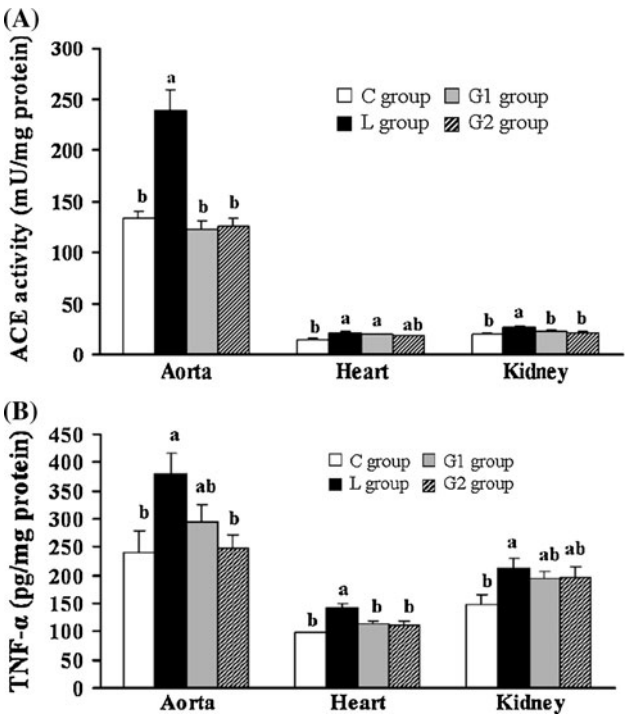

Fig. 2 ACE activity and TNF-α concentration in tissues in rats with L-NAME-induced hypertension. C, control group; L, L-NAME group; G1, rats fed 4 % chlorella; G2, rats fed 8 % chlorella. Values are presented as mean ± SEM. ($n = 8$). *Different superscript letters* in a row indicate a significant difference ($p < 0.05$)

Kidney cytochrome P450 4A expression and pathohistological analysis

To investigate the effects of chlorella on CYP4A expression, we used a Western blot analysis, and results are presented in Fig. 3a. We found significantly lower CYP4A expression in the G1 and G2 than in the L group.

Pathohistological analytical results of the heart are shown in Fig. 3. We found intimal thickening and fibrosis around the arterioles in the L group. Less vascular remodeling was found in the G1 and G2 groups. We defined an injury scale of inflammation and fibrosis with 0 as normal, 1 as mild, 2 as moderate, and 3 as severe. In Fig. 3b, chlorella consumption significantly reduced the injury scale of coronary arteries, ventricles, and septum of the heart.

In the analysis of the kidneys, we found reductions in Bowman's space, glomerular congestion, interstitial inflammation and hyperplastic changes in the small arteries (Fig. 3c). Chlorella consumption also ameliorated the inflammation and fibrosis in kidney tissues.

Discussion

In the present study, we found that L-NAME in drinking water induced hypertension in rats and both 4 and 8 %

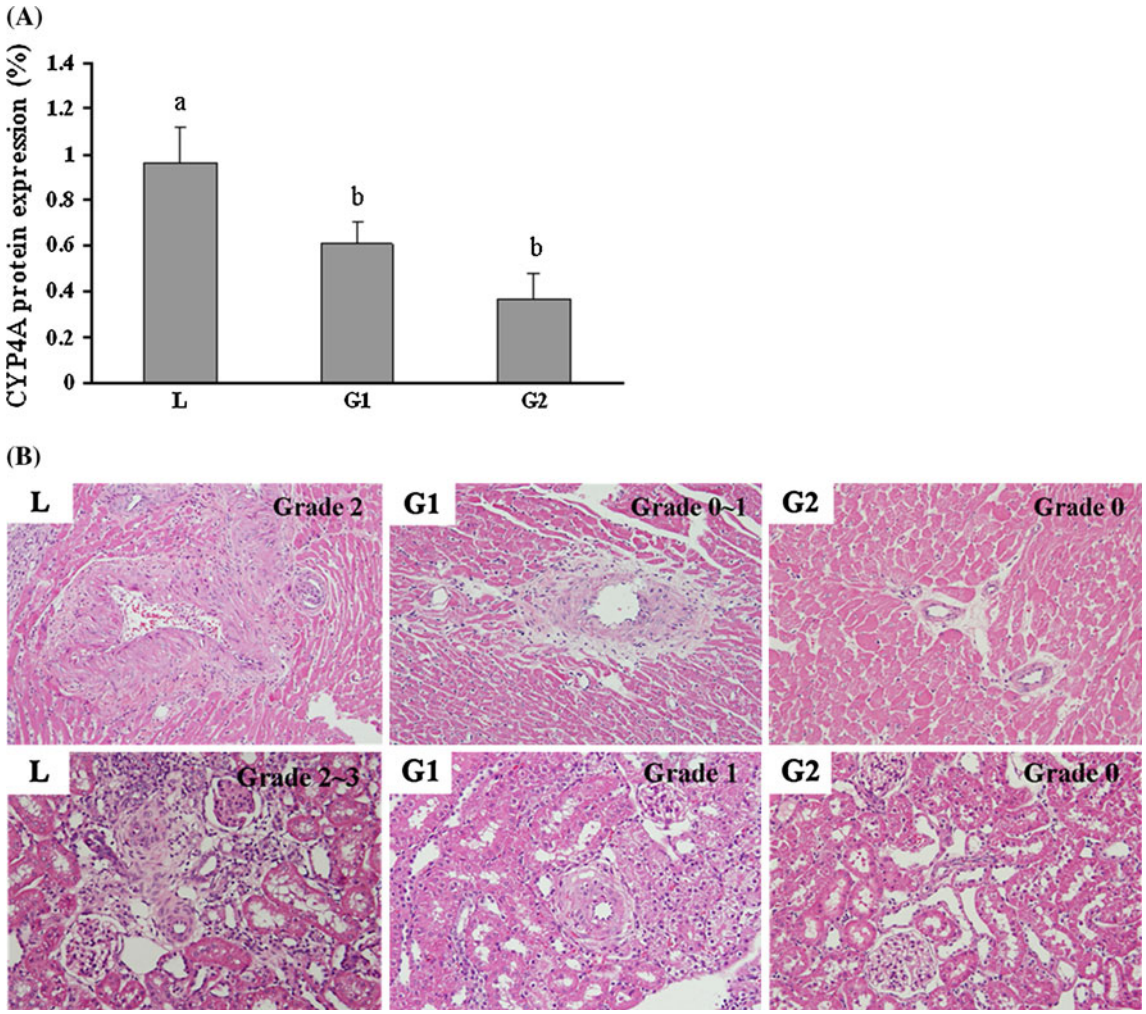

Fig. 3 CYP4A protein expression in microsomes from kidneys (**a**), and pathohistological analysis of hearts (**b**) and small arteries and glomerulus in kidneys (**c**). HE stain ×200. L, L-NAME group; G1, rats fed 4 % chlorella; G2, rats fed 8 % chlorella. Values are presented as mean ± SEM. ($n = 8$). Different *superscript letters* in a row indicate a significant difference ($P < 0.05$)

chlorella in the diet retarded the elevation of blood pressure and ameliorated inflammation and tissue damage in the heart and kidney. Rats consuming a diet containing 8 % chlorella had significantly lower DBP from the second week of the experiment. In addition, adding chlorella to the diet did not affect the growth or food intake of rats.

Hypertension may be related to an increase in oxidative stress in vivo [10, 11]. In rats with naphthalene-induced oxidative stress, treatment with 70 mg/kg of a chlorella extract lowered plasma, liver, and kidney MDA concentrations, and elevated plasma and tissue vitamin C and E levels [12]. Supplementation of the diet with 5 % chlorella also elevated liver antioxidative enzyme activities and reduced MDA concentrations [13]. However, we found that chlorella ameliorated L-NAME-induced hypertension, while no differences in either plasma or tissue MDA levels were found among the groups. Effects of antioxidant supplementation on hypertension remain controversial, and the outcomes may vary due to many factors such as the type, dosage, and duration of supplementation. Antioxidant therapy may also fail to show the expected effect due to individual differences [14]. In addition, reactions between superoxides and antioxidative vitamins were 5,000–10,000-fold slower than those between superoxides and NO [15]. Chronic inhibition of NO synthesis activates angiotensin II and increases production of superoxides and oxidative stress [16]. Therefore, we suggested that the antioxidants contained in chlorella might not be sufficient to improve the elevation in oxidative stress in rats with NO-deficiency induced hypertension.

L-Arginine is the precursor of NO, a vasodilator. Supplementation of arginine may improve endothelial dysfunction and lower blood pressure. Consumption of a diet rich in arginine was also reported to reduce blood pressure and increase the CCr in humans [17]. Studies also indicated that arginine intake may boost NO production in vivo [18].

Arginine is the most abundant amino acid in chlorella. In our study, daily arginine intake levels of each rat in the G1 and G2 groups were 76.2 and 152.4 mg, which equals to 1.27 and 2.54 g for healthy human adults. A study of hypertensive subjects showed that arginine supplementation of at least 12 g/day was needed to lower SBP and DBP [19]. Thus, the amount of arginine intake might not be sufficient to reduce blood pressure. In addition, we found no change in plasma arginine or NO_x concentrations in either chlorella group, while the elevation of blood pressure was retarded.

ACE inhibitors have been widely used to treat hypertension and were demonstrated to decrease the morbidity and mortality of cardiovascular and kidney diseases. ACE inhibition with perindopril improved endothelial dysfunction, decreased the von Willebrand factor, and increased endothelial NO synthase (eNOS) activity in patients with coronary heart disease [3]. In our study, chlorella dose-dependently inhibited ACE activity, and this was consistent with its effects on blood pressure. Other studies on L-NAME-induced hypertension also reported that the ACE inhibitors significantly reduced blood pressure [20]. In addition to the effect on blood pressure, ACE inhibitors are also tissue-protective. Treatment of NO-deficient rats with Enalapril produced improvements in renal vascular damage and reductions in urine proteins [21]. Guidelines of the American College of Cardiology (ACC)/American Heart Association (AHA) also suggested that ACE inhibitors should be used in the therapy of patients with high cardiovascular risks [22]. We found that chlorella may suppress the elevation of ACE activity induced by NOS inhibition, especially in the aorta and heart. Rats consuming chlorella also had lower urine protein levels and improved in cardiorenal remodeling. Various effects of ACE inhibition in the plasma and tissues may be related to the different affinities of ACE inhibitors with target organs. A previous study also showed that peptides composed of three to five amino acids derived from chlorella hydrolysates had ACE-inhibitory activity [23]. These results suggested that chlorella may be protective in cardiorenal remodeling through tissue ACE inhibition.

Chronic inhibition of NO may elevate plasma and tissue TNF-α and PAI-1 concentrations [24]. PAI-1 is related to the deposition of extracellular matrix and fibrotic proteins, which may result in thrombosis and fibrosis of vascular tissues. TNF-α impairs endothelial functions through stimulating the production of reactive oxygen species and reduction of NO bioavailability [25]. Chlorella is thought to exhibit anti-inflammatory activity. In rats fed on an atherogenic diet, 5 % of chlorella in the diet may cause repressed inflammatory factor activation [13]. In addition, ACE inhibitors were reported to suppress inflammation in NO-deficient rats. In clinical studies, ACE inhibitors also reduced TNF-α and angiotensin II levels in patients with cardiovascular disease [3 [26], and PAI-1 levels in hypertensive subjects [27]. These results suggested that chlorella may inhibit the activation of ACE and reduce PAI-1 and TNF-α concentrations.

Chronic inhibition of NO synthesis also promoted CYP4A expression and 20-HETE formation [28]. 20-HETE, the metabolite derived from actions of CYP4A, is vasoconstrictive and is important in the modulating of renal hemodynamics. In our study, we found that CYP4A protein expressions were lower in the 4 and 8 % chlorella groups compared to the L-NAME group. Consistently, we also observed that chlorella ameliorated L-NAME-induced inflammation, fibrosis and intimal thickening in the heart and kidneys. PAI-1 and TNF-α levels in the heart and kidneys were also lower in the chlorella groups. A study in NO-deficient rats demonstrated that inhibition of CYP4A expression may reduce mean arterial pressure and renal vascular resistance and increase the glomerular filtration rate [29]. Therefore, the renoprotective effects of chlorella may also be related to the modulation of CYP4A expression.

In conclusion, chlorella may inhibit the elevation of ACE activity, retard the development of hypertension and protect the cardiorenal system from injuries induced by chronic NO inhibition. Our findings of the beneficial effects of chlorella consumption in an endothelial dysfunction model might be important in modifying the diet to prevent early organ damage in people with high cardiovascular disease risks.

Acknowledgments All the authors have seen and approved the contents of the submitted manuscript. All the authors stated that there are no conflicts of interest, and all the authors adhere to the Committee on Publication Ethics guidelines on research and publication ethics. This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors. The contribution of each author was as follows: Yi-Ching Yang, Hsiang-Chi Peng, Pei-Yin Ho: doing experiments; Su-Ching Yang, Hsin-Yi Yang: writing of the manuscript and designing the study.

Conflict of interest All authors have no relevant conflict of interest to disclose.

References

1. Taddei S, Virdis A, Mattei P et al (1996) Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. Circulation 94:1298–1303
2. Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, Zatz R (1992) Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20:298–303
3. Ceconi C, Fox KM, Remme WJ et al (2007) ACE inhibition with perindopril and endothelial function. Results of a substudy of the EUROPA study: PERTINENT. Cardiovasc Res 73:237–246

4. Miranda MS, Sato S, Mancini-Filho J (2001) Antioxidant activity of the microalga *Chlorella vulgaris* cultered on special conditions. Boll Chim Farm 140:165–168
5. Sansawa H, Takahashi M, Tsuchikura S, Endo H (2006) Effect of chlorella and its fractions on blood pressure, cerebral stroke lesions, and life-span in stroke-prone spontaneously hypertensive rats. J Nutr Sci Vitaminol (Tokyo) 52:457–466
6. Merchant RE, Andre CA, Sica DA (2002) Nutritional supplementation with *Chlorella pyrenoidosa* for mild to moderate hypertension. J Med Food 5:141–152
7. Griesmacher A, Kindhauser M, Andert SE et al (1995) Enhanced serum levels of thiobarbituric-acid-reactive substances in diabetes mellitus. Am J Med 98:469–475
8. Miranda KM, Espey MG, Wink DA (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5:62–71
9. Shalaby SM, Zakora M, Otte J (2006) Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. J Dairy Res 73:178–186
10. Palm F, Nordquist L (2011) Renal oxidative stress, oxygenation, and hypertension. Am J Physiol Regul Integr Comp Physiol 301:R1229–R1241
11. Sugamura K, Keaney JF Jr (2011) Reactive oxygen species in cardiovascular disease. Free Radic Biol Med 51:978–992
12. Vijayavel K, Anbuselvam C, Balasubramanian MP (2007) Antioxidant effect of the marine algae *Chlorella vulgaris* against naphthalene-induced oxidative stress in the albino rats. Mol Cell Biochem 303:39–44
13. Lee HS, Choi CY, Cho C, Song Y (2003) Attenuating effect of chlorella supplementation on oxidative stress and NFkappaB activation in peritoneal macrophages and liver of C57BL/6 mice fed on an atherogenic diet. Biosci Biotechnol Biochem 67:2083–2090
14. Pechanova O, Simko F (2009) Chronic antioxidant therapy fails to ameliorate hypertension: potential mechanisms behind. J Hypertens 27(Suppl 6):S32–S36
15. Napoli C, Sica V, de Nigris F et al (2004) Sulfhydryl angiotensin-converting enzyme inhibition induces sustained reduction of systemic oxidative stress and improves the nitric oxide pathway in patients with essential hypertension. Am Heart J 148:e5
16. Kitamoto S, Egashira K, Kataoka C et al (2000) Chronic inhibition of nitric oxide synthesis in rats increases aortic superoxide anion production via the action of angiotensin II. J Hypertens 18:1795–1800
17. Siani A, Pagano E, Iacone R et al (2000) Blood pressure and metabolic changes during dietary L-arginine supplementation in humans. Am J Hypertens 13:547–551
18. Mann GE, Yudilevich DL, Sobrevia L (2003) Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. Physiol Rev 83:183–252
19. Ast J, Jablecka A, Bogdanski P et al (2010) Evaluation of the antihypertensive effect of L-arginine supplementation in patients with mild hypertension assessed with ambulatory blood pressure monitoring. Med Sci Monit 16:CR266–CR271
20. De Gennaro Colonna V, Rigamonti A, Fioretti S et al (2005) Angiotensin-converting enzyme inhibition and angiotensin AT1-receptor antagonism equally improve endothelial vasodilator function in L-NAME-induced hypertensive rats. Eur J Pharmacol 516:253–259
21. Pereira LM, Almeida JR, Mandarim-de-Lacerda CA (2004) Kidney adaptation in nitric oxide-deficient Wistar and spontaneously hypertensive rats. Life Sci 74:1375–1386
22. Vijan SG (2009) Angiotensin-converting enzyme inhibitors (ACEIs), not angiotensin receptor blockers (ARBs), are preferred and effective mode of therapy in high cardiovascular risk patients. J Indian Med Assoc 107:178–182
23. Suetsuna K, Chen JR (2001) Identification of antihypertensive peptides from peptic digest of two microalgae, *Chlorella vulgaris* and Spirulina platensis. Mar Biotechnol (NY) 3:305–309
24. Katoh M, Egashira K, Mitsui T et al (2000) Angiotensin-converting enzyme inhibitor prevents plasminogen activator inhibitor-1 expression in a rat model with cardiovascular remodeling induced by chronic inhibition of nitric oxide synthesis. J Mol Cell Cardiol 32:73–83
25. Zhang H, Park Y, Wu J et al (2009) Role of TNF-alpha in vascular dysfunction. Clin Sci (Lond) 116:219–230
26. Gonzalez W, Fontaine V, Pueyo ME et al (2000) Molecular plasticity of vascular wall during N(G)-nitro-L-arginine methyl ester-induced hypertension: modulation of proinflammatory signals. Hypertension 36:103–109
27. Fogari R, Zoppi A (2005) Is the effect of antihypertensive drugs on platelet aggregability and fibrinolysis clinically relevant? Am J Cardiovasc Drugs 5:211–223
28. Oyekan AO, Youseff T, Fulton D, Quilley J, McGiff JC (1999) Renal cytochrome P450 omega-hydroxylase and epoxygenase activity are differentially modified by nitric oxide and sodium chloride. J Clin Invest 104:1131–1137
29. Huang H, Zhou Y, Raju VT et al (2005) Renal 20-HETE inhibition attenuates changes in renal hemodynamics induced by L-NAME treatment in pregnant rats. Am J Physiol Renal Physiol 289:F1116–F1122