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ARTICLE

Morton Lentil Extract Attenuated Angiotensin II-Induced Cardiomyocyte Hypertrophy via Inhibition of Intracellular Reactive Oxygen Species Levels in Vitro

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The objective was to investigate whether a lentil (Morton) extract had any protective effect on cardiac hypertrophy, which is one of the most significant sequelae of cardiovascular diseases. High phenolic compounds (43.4 mg of GAE/g), including thirteen phenolic acid and two flavonoids, were detected in the acetone/water/acetic acid lentil extract. The extract showed strong antioxidant ability (105 μmol of TE/g). The effect of lentil extract on angiotensin (Ang) II-induced cardiac hypertrophy was examined. Results showed that pretreatment with lentil extract (25, 50, 100 μg/mL) significantly attenuated Ang II (0.1 μM)-induced hypertrophy by 18, 28, and 36% in rat cardiomyocytes, respectively; lentil extract (12.5, 25, 50 μg/mL) attenuated Ang II (0.1 μM)-induced hypertrophy by 9, 17, and 25% in human cardiomyocytes, respectively. Intracellular reactive oxygen species (ROS) levels were enhanced by Ang II treatment, and this stimulatory action was significantly attenuated (33% inhibition) by lentil extract (100 μg/mL) in rat cardiomyocytes and attenuated by 22% by 50 μg/mL lentil extract in human cardiomyocytes. In conclusion, Morton lentil extracts attenuated Ang II-induced rat and human cardiomyocytes hypertrophy via decreasing intracellular ROS levels.

KEYWORDS: Lentil; antioxidant; cardiomyocytes hypertrophy; reactive oxygen species

INTRODUCTION

Cardiac hypertrophy is one of the most significant sequelae of ischemic heart disease, hypertension, and valvular disease (*1*). Cardiac hypertrophy may develop in response to pressure and/or volume overload (*2*) and sustained hypertrophy can ultimately progress to ischemia, arrhythmia, heart failure, and sudden death (*3*). In patients, cardiac hypertrophy is a powerful and independent predictor of subsequent morbidity and mortality (*4*).

Angiotensin II (Ang II) is an important humoral factor responsible for cardiomyocyte hypertrophy (*5*) and is emerging as a key molecule both in the development of cardiac hypertrophy and in the pathogenesis of progressive myocardial dysfunction leading to heart failure (*6*). A large amount of evidence recently accumulated indicates that reactive oxygen species (ROS) function as intracellular messengers to modulate signaling pathways (*7*). An increase in ROS production in vivo is implicated in the Ang II-induced left-ventricular hypertrophy pathophysiology (*8*). Moreover, several studies reported that ROS generation is increased by Ang II treatment in cardiomyocytes and that inhibition of ROS generation by antioxidants abolishes Ang II-induced cardiomyocyte enlargement (*7*).

Legumes are an important source of foods, which supply the diet with complex carbohydrates, soluble fibers, essential vitamins, and metals, as well as polyphenols such as flavonoids, isoflavones, and lignans (*9*). Epidemiological studies have shown correlations between the consumption of foods with a high content of phenolics, including legumes, and decreasing incidence of cardiovascular diseases (*10*). Natural polyphenols, including simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans, and lignins, exert their beneficial health effects by their antioxidant activity (*11*). These compounds are capable of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, reducing α-tocopherol radicals, and inhibiting oxidases (*12*, *13*). Legumes possess different levels of antioxidant activities. As compared to beans, peas, and soybeans, lentils exhibit greater antioxidant activity (*14*, *15*).

Our objective in the present study was to determine the effect of a phenolic extract of lentil (Morton) on Ang II-induced hypertrophy and increases in intracellular ROS levels in rat and human cardiomyocytes, due to its high antioxidant ability. Accordingly, a diet containing lentils may help prevent cardiac hypertrophy and its related heart diseases.

MATERIALS AND METHODS

Lentil Materials and Extraction. Dry Morton lentil seeds provided by Spokane Seed Co. (Spokane, WA) were ground to flour with an IKA all basic mill (IKA Works Inc., Wilmington, NC), to pass through a 60-mesh sieve. Extraction procedures were described in our previous publication (*16*). Briefly, lentil flour was extracted twice with acetone/water/acetic acid (70:29.5:0.5, v/v/v) in 1:10 (w/v) at room temperature by stirring, first for 3 h and then overnight with another fresh solvent. The combined extract was concentrated by rotary evaporation at 40 °C, freeze-dried, and stored at −20 °C, until use.

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Total Phenolic Compound Analysis. The amount of total phenolics in Morton extract was determined by a Folin−Ciocalteu assay (*16*). A volume of 100 μL of extract (4 mg/mL), 200 μL of 10% (v/v) Folin−Ciocalteu's reagent, and 800 μL of 700 mM Na_2CO_3 was mixed and incubated at room temperature for 2 h. The absorbance was measured at 765 nm using a Multiscan Spectrum spectrophotometer (Thermo Electron Corp.). The total phenolic compound was expressed as milligrams of gallic acid equivalent per gram of extract (mg of GAE/g).

HPLC Analysis of Phenolic Compound Content. The quantitative analysis of free phenolic compounds was performed by using a 1200 Waters HPLC as described in our previous publication with a little modification (*15*). A 4.6 mm × 250 mm, 5 μm, A Zorbax Stablebond Analytical SB-C18 column (Agilent technologies, Rising Sun, MD) was used for the separation at 40 °C. Elution was performed using mobile phase A (0.1% TFA aqueous solution) and mobile phase B (100% methanol). The flow rate was set to 0.7 mL/min. The solvent gradient in volumetric ratios was as follows: Phase B was increased from 5 to 30% in 50 min and held at 30% for 15 min. Then phase B was increased to 100% at 67 min and then held at 100% for 10 min to clean up the column. Finally, phase B was decreased to 5% and held for 10 min to regenerate the column. The triplicate 6.25, 12.5, 25, and 50 μg/mL phenolic standards and Morton 20 mg/mL extract were analyzed at UV 270 and 325 nm.

All phenolic standards used in the analysis were purchased from Sigma (St. Louis, MO). They were gallic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), 2,3,4-trihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde acid (protocatechualdehyde acid), 2,5-dihydroxybenzoic acid (gentisic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), caffeic acid, chlorogenic acid, vanillin, syringic acid, 4-hydroxycinnamic acid (*p*-coumaric acid), syringaldehyde, 3-hydroxycinnamic acid (*m*-coumaric acid), *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid), salicylic acid, sinapic acid, 2-hydroxycinnamic acid (*o*-coumaric acid), 4-hydroxybenzoinic acid (*p*-hydroxybenzoic acid), *trans*-cinnamic acid, myricetin, 6-hydroxyflavone, apigenin, luteolin, (+)-catechin hydrate, and (−)-epicatechin.

Oxygen Radical Absorbance Capacity Assay (ORAC). Antioxidant capacity in the extract was measured by ORAC method as described in our previous study (*16*). In brief, 20 μL of extract (10 μg/mL) in phosphate buffer solution (PBS, 75 mM, pH 7.0), PBS blank, and 50, 25, 12.5, 6.25 μM Trolox standard in PBS solution was loaded to 96-well microplates in triplicate. The plate was then incubated in a FLUOstar OPTIMA Multifunction microplate reader (BMG Biotech, Durham, NC) at 37 °C for 40 min. Fluorescein solution (200 μL, 0.1 μM, Mallinckrodt Baker) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 20 μL, 3.2 μM) were added into appointed wells in a 96-well microplate. The kinetics of the fluorescence changes were recorded immediately by the software at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The final ORAC values were calculated using a linear equation between Trolox standards or sample concentration and net area under the fluorescence decay curve and were expressed as micromoles of Trolox equivalent per gram of extract sample (μmol of TE/g).

Cardiomyocyte Culture. Primary cultured cardiomyocytes were prepared from neonatal rats according to previously published procedures with some modifications (*17*). In brief, 1-day-old Sprague−Dawley rats (Charles River Laboratories International, Wilmington, MA) were anesthetized with sodium pentobarbital (200 mg/kg ip, Sigma, St. Louis, MO). Ventricles were minced in cold Hank's buffered salt solution (HBSS, BioWhittaker, Walkersville, MD) and washed one additional time. The minced tissue was digested with 0.25% trypsin (Media Technology, Oklahoma City, OK) in a 37 °C water bath with shaking for 10 min. Isolated cells were suspended in Dulbecco's modification of Eagle's medium (American Type Culture Collection, Manassas, VA) composed of 10% fetal bovine serum and 1% penicillin−streptomycin (BioWhittaker) at 37 °C for 1 h in a humidified atmosphere with 95% O_2 and 5% CO_2 to let fibroblast attach to the bottom of the plate. Suspended cell solution (1×10^5 cells/well) was transferred to a 6-well plate for morphologic study and to a 24-well plate for measurement of ROS levels. The cardiomyocytes were cultured for an additional 48 h.

Human cardiomyocytes, which were purchased from Promocell (Heidelberg, Germany), were isolated from normal human ventricular tissue of the adult heart and were cryopreserved at passage 2. They were cultured in myocyte growth medium (Promocell) at 37 °C in a humidified atmosphere with 95% O_2 and 5% CO_2. The cells were seeded in 24- or 6-well plates (2×10^4 cells/well) for 72 h (∼70−90% confluency) before being used for assays.

Immunocytochemistry and Measurement of Cell Surface Area. Cultured cardiomyocytes were pretreated with HBSS, Morton extract (12.5, 25, 50, or 100 μg/mL), or antioxidant butylated hydroxyanisole (BHA, 12.5, 25, and 50 μM, Sigma) for 24 h. Ang II (100 nM) was then added into the culture media and cultured for an additional 24 h. Each treatment was performed in triplicate wells. Monoclonal anti-α-sarcomeric actin antibody (Sigma, St. Louis, MO) was used for the identification and measurement of the cardiomyocyte via immunofluorescence staining. In brief, cells were fixed in methanol at −10 °C and washed with fresh washing solution (0.1% Triton X-100 in PBS) three times. After preincubation with 3% of bovine serum albumin (BSA) for 20 min, the cells were incubated with primary antibody, which was diluted to 1:100 with PBS containing 3% BSA, overnight at 4 °C. After three washings with the washing solution, the cells were incubated with fluorescence-conjugated anti-mouse IgG (1:500 diluted in PBS) for 1 h at room temperature.

Cell surface area was measured according the published study with some modifications (*18*). The photo images of cardiomyocytes (magnification ×100 for human cardiomyocytes and ×400 for rat cardiomyocytes) were taken by a fluorescence microscope. The cell surface area of positive image for sarcomeric-actin staining cardiomyocytes was measured by the image analysis software (Image Pro plus 6.0, Media Cybernetics, Bethesda, MD). The variation of cell size was expressed as relative cell surface area versus the control. At least 50 cells were randomly selected for surface area analysis.

Measurement of Intracellular ROS Generation. ROS generation was determined using two oxidant-sensitive fluorogenic probes. Dihydroethidium (DHE; excitation/emission wavelength: 485 nm/590 nm; Molecular Probes) (*17*), sensitive to superoxide, can be oxidized by ROS, including $O^{\bullet}$ and/or hydroxyl radicals to yield fluorescent ethidium (Eth). Eth binds to DNA (Eth-DNA), further amplifying its fluorescence. Thus, the increases in Eth-DNA fluorescence are suggestive of $O^{\bullet}$ generation (*19*). Another fluorogenic probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA' excitation−emission wavelengths, 485 and 538 nm; Molecular Probes), sensitive to H_2O_2 and hydroxyl radicals, can be oxidized by ROS to yield the fluorescent product DCF. Thus, the increases in DCF fluorescence suggest H_2O_2 or hydroxyl radical generation (*19*).

After treatment with HBSS control, Ang II (100 nM), or Ang II plus Morton extract or BHA as described above, the cultured cardiomyocytes were loaded with DHE or DCFH-DA. The intracellular ROS levels were measured using a fluorometric imaging plate reader (Spectra Max Gemini EM, Molecular Devices Corp.) to detect changes in fluorescence resulting from intracellular probe oxidation.

Statistical Analysis. Data are expressed as mean ± SD. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Kruskal−Wallis ANOVA on ranks for multivariance. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Chemical Characteristics and Antioxidant Ability of Morton Extract. In the present study, 1.023 g of phenolic extract was produced from 10 g of Morton flour using acetone/water/acetic acid solvent, which contained about 43 mg/g of total phenolic compounds expressed as gallic acid. The results of HPLC (**Table 1**) showed some similarities with our previous analysis of the individual phenolic compounds contained in the extract (*15*) that (+)-catechin, (−)-epicatechin, and sinapic acid are the major phenolic substances in lentils. However, our analysis also showed a high salicylic acid content. This might be due to our modification of the extraction method by using a strong stirring device. Our previous extraction method (*15*) using an orbital shaker was much gentler than this stirring method. ORAC in the Morton extract showed a high antioxidant capacity (105 μM Trolox/g), which was higher than the 95 μM Trolox/g observed in our previous study (*20*), in which the sample was extracted using the gentle orbital shaker. The difference in ORAC could be attributed to the compositional differences in the phenolic compounds extracted.

Table 1. Phenolic Compounds Identified in Morton Acetone/Water/Acetic Acid Extracts

chemical compound	content (μg/g, mean ± SD)
(+)-catechin hydrate	17270.4 ± 524.1
(−)-epicatechin	5319.8 ± 87.0
salicylic acid	17784.2 ± 93.3
sinapic acid	5296.9 ± 82.2
vanillic acid	410.4 ± 66.3
syringic acid	244.6 ± 26.6
vanillin	201.4 ± 3.8
gallic acid	756.7 ± 29.3
ferulic acid	679.2 ± 92.2
chlorogenic acid	537.9 ± 78.6
o-coumaric acid	192.3 ± 2.8
p-hydroxybenzoic	40.5 ± 17.3
protocatechuic acid	280.2 ± 25.2
protocatechualdehyde	123.6 ± 23.0
2,3,4-trihydroxybenzoic acid	150.3 ± 28.0

Attenuation of Ang II-Induced Rat Ventricular Cardiomyocyte Hypertrophy by the Morton Extracts. Exposure of cardiomyocytes to 100 μg/mL Morton extracts for 48 h did not significantly alter the size of myocardial cells (**Figure 1Ab**) as compared to the control (HBSS treatment, **Figure 1Aa**,**B**). Ang II (0.1 μM) treatment for 24 h induced significant cardiomyocyte hypertrophy (**Figure 1Ac**,**B**). Measurement of cell surface area revealed an increase of 50.7 ± 3.3% in response to Ang-II as compared to the control ($p < 0.001$, **Figure 1B**). However, treating cells with Morton extract significantly alleviated Ang II-induced hypertrophic response (**Figure 1 Ad**). Cell surface area was decreased by 13.6 ± 2.6, 27.5 ± 1.7 ($p < 0.05$), and 36.2 ± 1.1% ($p < 0.001$) after treatment with Morton extract (25, 50, and 100 μg/mL, respectively), as compared with Ang II treatment alone (**Figure 1B**). Taken together, the present data demonstrate that Morton extract attenuates Ang II-induced cardiomyocyte hypertrophy in a dose-dependent manner.

Reduction of Ang II-Induced Cellular ROS Stress in Cultured Neonatal Rat Ventricular Cardiomyocytes by Morton Extracts. Previous studies from other research groups demonstrated that angiotensin II increased the generation of ROS in cardiomyocytes (*21*). These results were supported by our observations showing that Ang II treatment significantly increased intracellular $O^{\bullet}$ and/or hydroxyl radical generation by 76.7 ± 2.2% and H_2O_2 and/or hydroxyl radicals by 73.4 ± 3.8% as compared to the control (**Figure 2A**,**B**). Our previous (*14*, *15*) and present studies showed that Morton extract was rich in polyphenolic compounds and possessed high antioxidant capability. Treatments of cardiomyocytes with 25, 50, and 100 μg/mL Morton extract for 24 h significantly inhibited Ang II-induced increases in intracellular $O^{\bullet}$ and/or hydroxyl radical levels by 13.2 ± 3.1 ($p < 0.05$), 32.5 ± 1.4 ($p < 0.05$), and 41.3 ± 0.9% ($p < 0.001$), respectively, and also decreased Ang II-induced increases in H_2O_2 and/or hydroxyl radical levels by 12.6 ± 2.1 ($p < 0.05$), 30.6 ± 1.3 ($p < 0.05$), and 44.6 ± 0.8% ($p < 0.001$), respectively, as compared to the Ang II treatment alone (**Figure 2A**,**B**). However, the levels of $O^{\bullet}$, H_2O_2, and/or hydroxyl radical were not significantly decreased by100 μg/mL Morton extract alone (without Ang II treatment). In summary, these results clearly demonstrated that Morton extract dose-dependently attenuated angiotensin II-induced ROS increases in ventricular cardiomyocytes cultured from neonatal rats.

Attenuation of Ang II-Induced Human Cardiomyocyte Hypertrophy by Morton Extracts. Similar to the response in rat cardiomyocytes, exposure of human cardiomyocytes to 50 μg/mL Morton extracts for 48 h did not induce significant decrease in cell size (**Figure 3Ab**) as compared to the control (HBSS treatment; **Figure 3Aa**,**B**). Ang II (0.1 μM) treatment for 24 h induced significant cardiomyocyte hypertrophy (**Figure 3Ac**). The cell surface area was significantly increased by 32.3 ± 3.6% after treatment with Ang II, as compared to the control ($p < 0.001$, **Figure 3B**), whereas Morton extract significantly alleviated Ang II-induced hypertrophic response (**Figure 3 Ad**). The cell surface area was decreased by 8.6 ± 2.3, 19.1 ± 1.8 ($p < 0.05$), and 25.8 ± 2.1% ($p < 0.001$) after treatment with Morton extract (12.5, 25, and 50 μg/mL Ang II treatment, respectively), as compared with Ang II treatment alone (**Figure 1B**). In summary, the results demonstrated that Morton extract also dose-dependently attenuated Ang II-indued hypertrophy in human cardiomyocytes.

Reduction of Morton Extracts to Ang II-Induced Cellular ROS Stress in Human Cardiomyocytes. Treatment of human cardiomyocytes with Morton extract (50 μg/mL, 24 h) alone did not

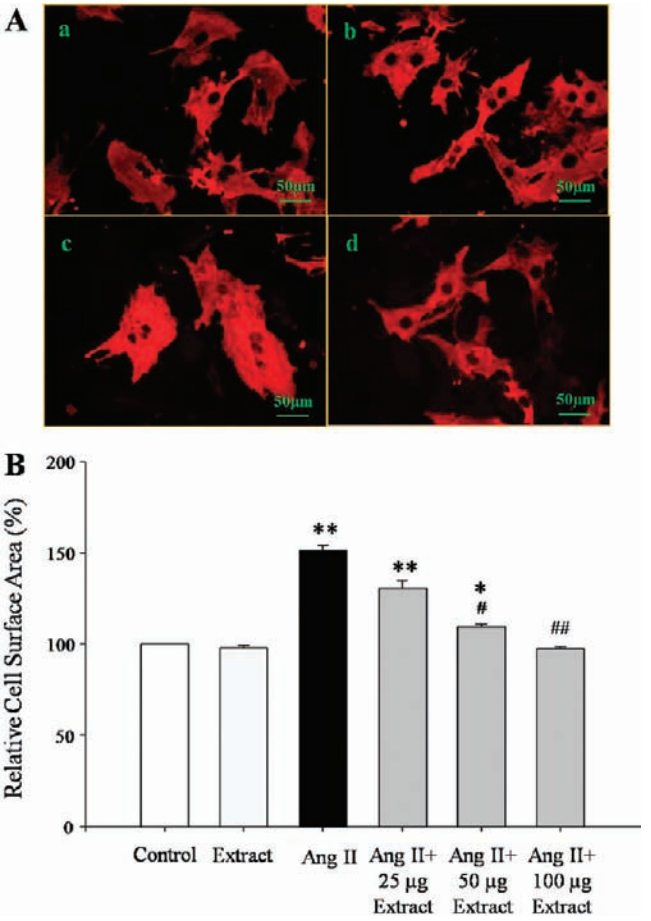

Figure 1. Effect of Morton extract on Ang II-induced cardiac hypertrophy in rat cardiomyocytes. (**A**) Representative fluorescence micrographs of cultured cardiomyocytes stained with α-sarcomeric actin antibody after the following treatments: control (HBSS, 48 h) (**a**); Morton extract alone (100 μg/mL, 48 h) (**b**); Ang II (0.1 μM, 24 h) alone (**c**); and Morton extract (48 h) plus Ang II (24 h) (**d**). (**B**) Bar graph summarizing the size of cardiomyocytes after the following treatments: control (HBSS, n = 50), Morton extract (100 μg/mL, n = 58) alone, Ang II (0.1 μM, n = 50) alone, and Ang II plus Morton extract at the concentrations of 25 μg/mL (n = 57), 50 μg/mL (n = 65), and 100 μg/mL (n = 63), respectively. Numbers in parentheses represent the number of cardiomyocytes used for quantification of the cell surface area. Data are means ± SD and were derived from three experiments and at least triplicate wells in each experiment. *, $p \leq 0.05$; or **,$p \leq 0.001$, significantly different from the control treatment; #, $p \leq 0.05$; or ##, $p \leq 0.001$, significantly different from the treatment with Ang II alone.

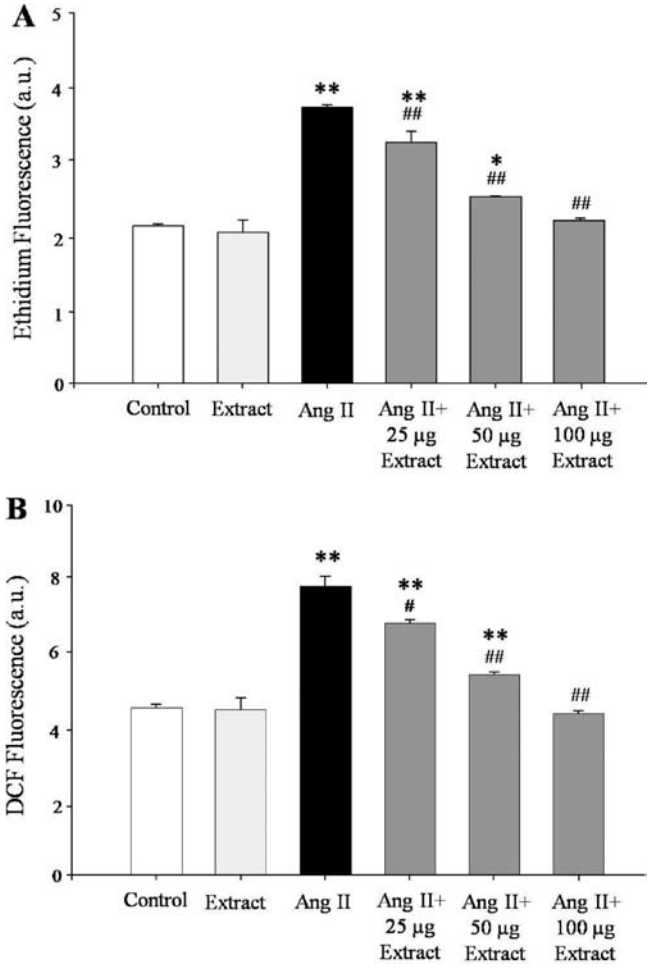

Figure 2. Effect of Morton extract and Ang II on intracellular ROS levels in rat cardiomyocytes. ROS levels were detected using oxidant-sensitive fluorogenic probes DHE (**A**) and DCFH (**B**) in rat cardiomyocytes as described under Materials and Methods. Cardiomyocytes were treated with control (HBSS), Morton extract, Ang II (100 nM), or Ang II plus Morton extract (25, 50, and 100 μg/mL), respectively. Fluorescence density of ethidium or DCF was immediately measured after addition of Ang II. Data are mean ± SD and derived from three experiments and at least triplicate wells in each experiment. $*$, $p \leq 0.05$; or $**$,$p \leq 0.001$, significantly different from the control (HBSS); #, $p \leq 0.05$; or ##, $p \leq 0.001$, significantly different from the treatment with Ang II alone.

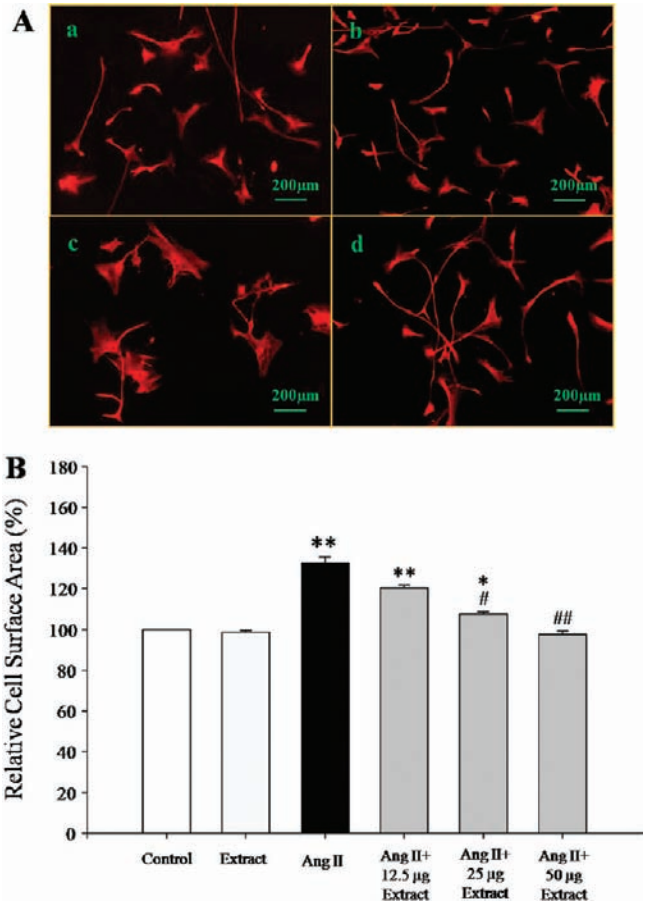

Figure 3. Effect of Morton exract on Ang II-induced cardiac hypertrophy in human cardiomyocytes. (**A**) Representative fluorescence micrographs of human cardiomyocytes stained with α-sarcomeric actin antibody after the following treatments: control (HBSS, 48 h) (**a**); Morton extract alone (50 μg/mL, 48 h) (**b**); Ang II (0.1 μM, 24 h) alone (**c**); or Morton extract (48 h) plus Ang II (24 h) (**d**) as described under Materials and Methods. (**B**) Bar graph summarizing the size of human cardiomyocytes after the following treatments: control (HBSS, $n = 40$), Morton extract (50 μg/mL, $n = 44$) alone, Ang II (0.1 μM, $n = 41$) alone, and Ang II plus Morton extract at the concentrations of 12.5 μg/mL ($n = 50$), 25 μg/mL ($n = 56$), and 50 μg/mL ($n = 53$), respectively. Numbers in parentheses represent the numbers of cardiomyocytes used for quantification of the cell surface area. Data are means ± SD and derived from three experiments at least triplicate wells in each experiment. $*$, $p \leq 0.05$; or $**$, $p \leq 0.001$, significantly different from the control treatment; #, $p \leq 0.05$; or ##, $p \leq 0.001$, significantly different from the treatment with Ang II alone.

significantly alter intracellular levels of $O^{\bullet}$, H_2O_2, and/or hydroxyl radical. However, treatment with Ang II (0.1 μM, 24 h) significantly increased intracellular $O^{\bullet}$ and/or hydroxyl radical generation by 25.9 ± 3.1% and H_2O_2 and/or hydroxyl radical generation by 22.4 ± 2.7%, as compared to the control (**Figure 4A**,**B**). Ang II-induced increases in $O^{\bullet}$ and/or hydroxyl radical levels were significantly inhibited by 8.9 ± 0.6 ($p < 0.05$), 11.7 ± 0.4 ($p < 0.05$), and 18.2 ± 1.3% ($p < 0.001$) after treatments with 12.5, 25, and 50 μg/mL Morton extract, respectively, as compared with Ang II treatment alone. In contrast, Ang II-induced increases in H_2O_2 and/or hydroxyl radical levels were attenuated by 8.9 ± 0.7 ($p < 0.05$), 11.3 ± 1.1 ($p < 0.05$), and 17.8 ± 1.4% ($p < 0.001$) after treatment with 12.5, 25, and 50 μg/mL Morton extract, respectively, as compared to Ang II treatment alone (**Figure 4A**,**B**). These data indicated that in both rat and human cardiomyocytes, Morton extract reduced angiotensin II-induced ROS stress dose-dependently.

Attenuation of BHA to Ang II-Induced Neonatal Cultured Rat Ventricular Cardiomyocytes Hypertrophic Response. BHA, a known antioxidant acting as a free radical scavenger, was used as the positive control. Exposure of cardiomyocytes to 50 μM BHA alone for 48 h did not induce significant effect on cell size (**Figure 5Ab**) as compared to the control (HBSS treatment; **Figure 5Aa**,**B**). However, treatments with 0.1 μM Ang II for 24 h induced significant cardiomyocyte hypertrophy (**Figure 5A**). Measurement of cell surface area revealed an increase of 56.9 ± 2.9% in response to Ang II as compared to the control ($p < 0.001$; **Figure 5B**), whereas BHA significantly alleviated Ang II-induced hypertrophic response (**Figure 5Ad**). Cell surface area decreased by 33.6 ± 2.4, 38.1 ± 1.8 ($p < 0.05$), and 38.5 ± 1.5% ($p < 0.001$) in Ang II plus 12.5, 25, and 50 μM BHA treatment groups, respectively, as compared to Ang II treatment alone, respectively (**Figure 5B**). In short, similar to the effect of Morton extract, BHA suppressed Ang II-induced cardiomyocyte hypertrophy dose-dependently.

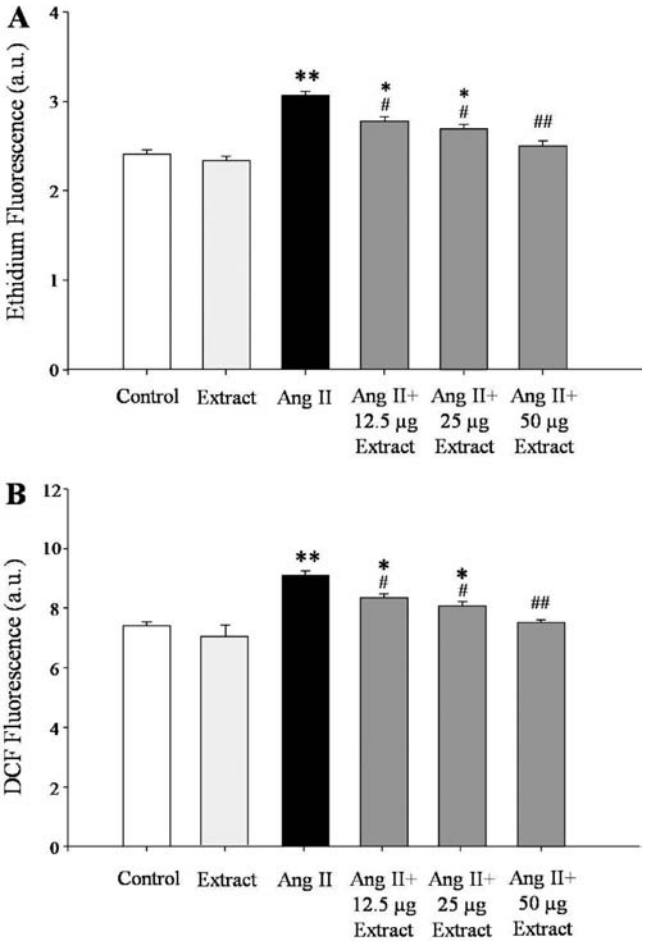

Figure 4. Effect of Morton extract and Ang II on intracellular ROS levels in human cardiomyocytes. ROS levels were detected using oxidant-sensitive fluorogenic probes DHE (**A**) and DCFH (**B**) in human cardiomyocytes. Cardiomyocytes were treated under the following conditions: control (HBSS), Morton extract (50 μg/mL), Ang II (100 nM), or Ang II plus Mortaon at the concentrations indicated in the figure (12.5, 25, 50 μg/mL). Fluorescence density of ethidium or DCF was immediately measured after the addition of Ang II. Data are mean $\pm$ SD and derived from three experiments and at least triplicate wells in each experiment. $*$, $p \leq 0.05$; or $**$,$p \leq 0.001$, significantly different from the control (HBSS); #, $p \leq 0.05$; or ##, $p \leq 0.001$, significantly different from the treatment with Ang II alone.

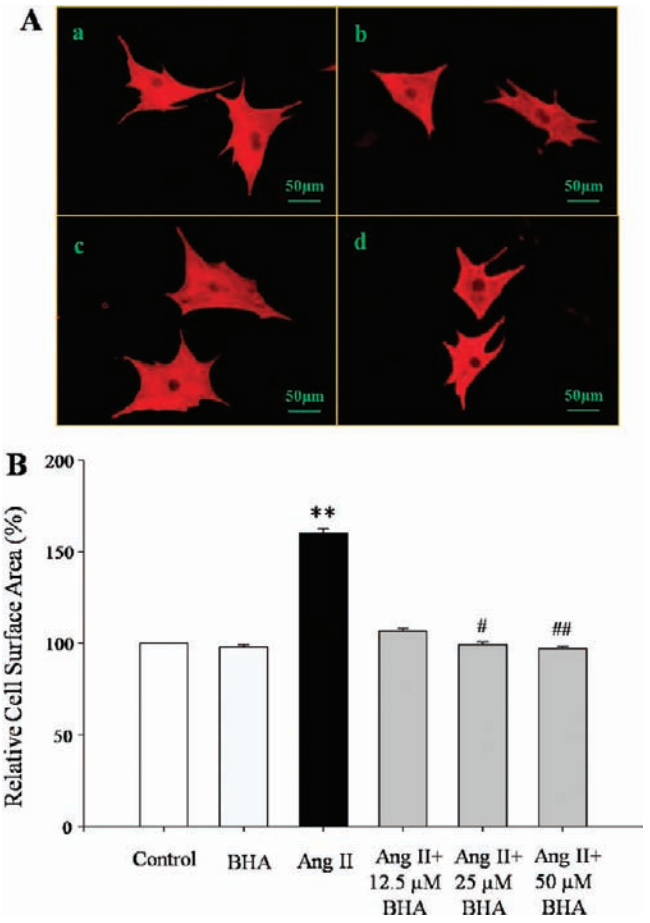

Figure 5. Effect of BHA on Ang II-induced cardiac hypertrophy in rat cardiomyocytes. (**A**) Representative fluorescence micrographs of cultured cardiomyocytes stained with α-sarcomeric actin antibody after the following treatments: control (HBSS, 48 h) (**a**); BHA alone (50 μM, 48 h) (**b**); Ang II (0.1 μM, 24 h) alone (**c**); and Ang II (24 h) plus BHA (48 h) at the concentrations (in μM) indicated in the figures. (**d**). (**B**) Bar graph summarizing the size of cardiomyocytes after the following treatments: control (HBSS, $n = 50$), BHA (50 μM, $n = 53$) alone, Ang II (0.1 μM, $n = 45$) alone, and Ang II plus BHA at concentrations of 12.5 μM ($n = 56$), 25 μM ($n = 60$), and 50 μM ($n = 55$). Numbers in parentheses represent the number of cardiomyocytes used for quantification of the cell surface area. Data are mean $\pm$ SD and derived from three experiments and at least triplicate wells in each experiment. $**$, $p \leq 0.001$, significantly different from the control treatment; #, $p \leq 0.05$; or ##, $p \leq 0.001$, significantly different from the treatment with Ang II alone.

Reduction of BHA to Ang II-Induced Cellular ROS Stress in Cultured Ventricular Cardiomyocytes from Neonatal Rats. Treatment with 0.1 μM Ang II significantly increased intracellular $O^{\bullet}$ and/or hydroxyl radical generation by $18.8 \pm 4.1\%$ and increased H_2O_2 and/or hydroxyl radical by $14.1 \pm 2.3\%$, as compared to the control (**Figure 6A**,**B**). Treatments with 12.5, 25, and 50 μM BHA inhibited intracellular $O^{\bullet}$ and/or hydroxyl radical levels by 4.5 ± 0.5, 8.4 ± 0.7 ($p < 0.05$), and $11.9 \pm 0.6\%$ ($p < 0.001$), respectively, and reduced H_2O_2 and/or hydroxyl radicals by 4.7 ± 0.6, 5.5 ± 0.7 ($p < 0.05$), and $12.3 \pm 1.0\%$ ($p < 0.001$), respectively, as compared to the Ang II treatment alone (**Figure 6A**,**B**). However, the levels of $O^{\bullet}$, H_2O_2, and/or hydroxyl radicals were not significantly affected by treatment with 50 μM BHA alone. The results of this study demonstrated that BHA dose-dependently inhibited angiotensin II-induced ROS increases in ventricular cardiomyocytes cultured from neonatal rats.

DISCUSSION

The major findings of the present study are as follows: (1) The phenolic extract from Morton was proven to contain high total phenolic compound and high antioxidant capacities. (2) Treatment with 0.1 μM Ang II for 24 h induced significant hypertrophy in rat and human cardiomyocytes. Cellular ROS, including $O^{\bullet}$, H_2O_2, and hydroxyl radical levels, increased significantly when cardiomyocytes were exposed to Ang II. (3) Treatments of rat or human cardiomyocytes with Morton extract did not alter cell size and intracellular cellular ROS levels. (4) Concurrent treatments with Morton extract and Ang II significantly alleviated Ang II-induced hypertrophic response and reduced Ang II induced increases in ROS levels. (5) As a positive control, BHA showed a significant inhibitory effect on Ang II-induced hypertrophy and ROS increases, mimicking the action of the Morton extract in cardiomyocytes.

Our previous studies showed that lentils exhibited much higher antioxidant activity as compared to common beans, peas, and yellow soybeans. Furthermore, phenolics content was correlated with antioxidant activity (*14*, *15*). The result that Morton lentil

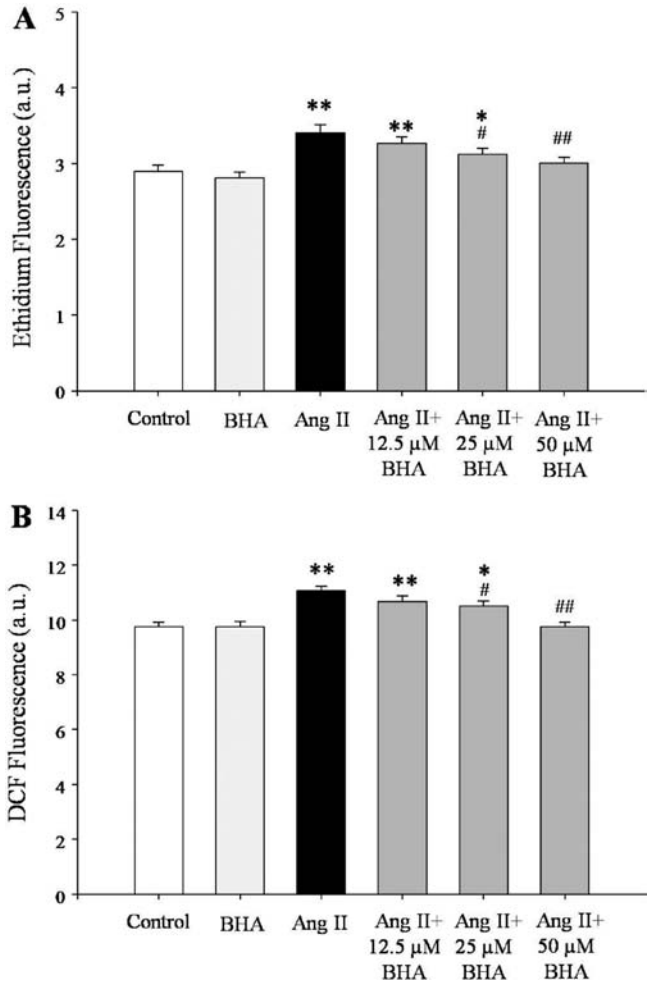

Figure 6. Effect of BHA and Ang II on intracellular ROS levels in rat cardiomyocytes. ROS levels were detected using oxidant-sensitive fluorogenic probes DHE (**A**) and DCFH (**B**) in rat cardiomyocytes as described under Materials and Methods. Cardiomyocytes were exposed to control (HBSS) or BHA (12.5, 25, and 50 μM) for 24 h, prior to the addition of Ang II (100 nM). Fluorescence density of ethidium or DCF was immediately measured after the addition of Ang II. Data are mean $\pm$ SD and derived from three experiments and at least triplicate wells in experiment. $*$, $p \leq 0.05$; or $**$, $p \leq 0.001$, significantly different from the control (HBSS); #, $p \leq 0.05$; or ##, $p \leq 0.001$, significantly different from the treatment with Ang II alone.

extract used in this study contained high total phenolics and high antioxidant capacities was in accordance with the previous findings. Our research suggested that the phenolic compounds in the extract exerted their beneficial health effects by their capability in removing free radicals, chelating metal catalysts, activating antioxidant enzymes, reducing α-tocopherol radicals, and inhibiting oxidases (*11*−*13*).

Angiotensin II-induced animal and human cardiomyocyte hypertrophy is a common model to study the mechanism and the effect of chemical drugs on myocardial hypertrophy in pharmacology (*2*, *22*). However, the use of this method for the study of dietary component effect on cardiomyocyte hypertrophy, particularly with respect to legume consumption, has not been reported. In the present study this model was utilized to investigate the effect of Morton extract on angiotensin II-induced cardiomyocyte hypertrophy. The Morton extract treatment alone did not produce significant effects on rat and human cardiomyocytes. The observations that treatment of cardiomyocytes with Morton extract or a known antioxidant BHA decreased Ang II-induced oxidative stress and prevented Ang II-induced cardiomyocyte hypertrophy strongly support the hypothesis that Ang II-induced cardiac hypertrophy is mediated mainly by oxidative stress. The results were consistent with previous observations on neonatal cardiomyocytes (*21*, *23*), demonstrating that Ang II increased NAD(P)H oxidase-dependent production of $O^{\bullet}$. The O^{-} formed can be rapidly converted to hydrogen peroxide, hypochlorous acid, hydroxyl radical, and other toxic species, which collectively are termed ROS. Because the lentil extract is high in antioxidant capacity, the inhibitory effect of Morton extract on Ang II-induced cardiomyocyte hypertrophy could be mediated by lowering intracellular ROS levels in both human and rat myocardial cells. However, how Morton extract may reduce intracellular ROS levels via inhibition of the activity of NAD(P)H oxidase, a central mediator of Ang II-induced cardiomyocyte hypertrophy (*24*), and how Morton extract may chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidase (*11*−*13*) have not been tested.

BHA, an antioxidant frequently used as a positive control in cardiomyocyte hypertrophy research (*25*), showed a significant effect in reducing Ang II-induced hypertrophy and ROS levels, mimicking the action of the Morton extract on rat cardiomyocytes. In addition, there was other evidence showing that some antioxidant chemicals such as trilinolein from a Chinese herb and propofol can partially block ROS formation and hypertrophic responses induced by Ang II in cardiomyocytes (*26*, *27*). An in vivo study showed that green tea extract, containing catechins that had antioxidant properties, attenuated Ang II-induced cardiac hypertrophy in rats via decreasing the expression of the NAD(P)H oxidase subunit gp91phox and the translocation of Rac-1, as well as NAD(P)H oxidase enzymatic activity (*28*). In addition, chlorogenic acid, ferulic acid, and caffeic acid had been proven to inhibit ROS generating enzymes including NAD(P)H and xanthine oxidase (*29*, *30*). Morton contained high contents of catechins and significant amounts of chlorogenic acid and ferulic acid (**Table 1**). Therefore, similar antioxidative mechanisms may occur in the inhibitory effect of Morton extract on Ang II-induced cardiomyocyte hypertrophy. However, the exact molecular and cellular mechanisms underlying Morton-induced reduction in intracellular ROS levels need to be further investigated.

The antioxidative effect of the Morton extract treatment alone was not accompanied by a significant change in the size of rat and human cardiomyocytes. These results suggest that the reduction of $O^{\bullet}$ below the basal level had no effect on cardiomycyte function, probably because superoxide dismutases and other endogenous scavengers of $O^{\bullet}$ successfully keep the basal oxidative reaction at a minimum under normal physiological conditions (*23*). In addition, our research indicated the prevention of Ang II-induced cardiomyocyte hypertrophy is likely to be mediated by the antioxidative property of Morton extract. These observations thus demonstrate that the antioxidative property of Morton extract might play an important role in protecting cardiac hypertrophy and other cardiac diseases. In addition to the antioxidative property, many other components such as significant amounts of folate and magnesium in lentils may also help decrease the risk factors for heart disease (*31*, *32*).

Normal human cardiomyocytes, used in the present study, are isolated from the ventricles of the adult heart and are qualified for in vitro research on cardiac diseases such as hypertrophy. This study provided the first evidence that Morton extract has the ability to attenuate not only Ang II-induced rat cardiomyocyte hypertrophy but also Ang II-induced human cardiomyocyte hypertrophy. The results directly support the hypothesis that Morton lentil may have beneficial effect in the prevention of human cardiac hypertrophy and its related heart diseases as a

pharmaceutical ingredient. Our findings are in accordance with epidemiologic evidence and experimental results in humans that showed a legume-containing diet was associated with a large 82% reduction in the risk of death from heart disease. People eating legumes had 22% less coronary heart disease and 11% less cardiovascular disease as compared to those who ate a normal diet in the United States (*31*).

In conclusion, exposure to Ang II significantly increased the production of $O^{\bullet}$ and induced hypertrophy in rat and human cardiomyocytes. Oxidative stress appears to play a major role in Ang II-induced cardiac hypertrophy. Morton extract significantly prevented the Ang II-induced cardiac effect, presumably through its antioxidative properties. These observations provide a new insight into the understanding of the cardiac benefits of Morton lentil in cardiac diseases. Our findings thus suggest that the inclusion of Morton lentil in the diet may have benefit to a variety of cardiac diseases, which are characterized by an increase in oxidative stress or by the activation of the rennin−angiotension system in humans.

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