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Roasted Coffees High in Lipophilic Antioxidants and Chlorogenic Acid Lactones Are More Neuroprotective than Green Coffees

Yi-Fang Chu,*,† Peter H. Brown,† Barbara J. Lyle,† Yumin Chen,† Richard M. Black,† Claire E. Williams,‡ Yi-Ching Lin,§ Chih-Wei Hsu,§ and Irene H. Cheng*,§,∥

†Kraft Foods Research & Nutrition, 801 Waukegan Road, Glenview, Illinois 60025, ‡Kraft Foods International, Ruscote Avenue, Banbury, Oxon, OX16 2QU, U.K., §Institute of Brain Science, National Yang-Ming University, 155 Li-Nong Street, Section 2, Peitou, Taipei, Taiwan, and ∥Brain Research Center, National Yang-Ming University, 155 Li-Nong Street, Section 2, Peitou, Taipei, Taiwan

Oxidative stress is involved in many neurodegenerative processes leading to age-related cognitive decline. Coffee, a widely consumed beverage, is rich in many bioactive components, including polyphenols with antioxidant potential. In this study, regular and decaffeinated samples of both roasted and green coffee all showed high hydrophilic antioxidant activity in vitro, whereas lipophilic antioxidant activities were on average 30-fold higher in roasted than in green coffee samples. In primary neuronal cell culture, pretreatment with green and roasted coffees (regular and decaffeinated) protected against subsequent H_2O_2-induced oxidative stress and improved neuronal cell survival (green coffees increased neuron survival by 78%, compared to 203% by roasted coffees). All coffee extracts inhibited ERK1/2 activation, indicating a potential attenuating effect in stress-induced neuronal cell death. Interestingly, only roasted coffee extracts inhibited JNK activation, evidencing a distinctive neuroprotective benefit. Analysis of coffee phenolic compounds revealed that roasted coffees contained high levels of chlorogenic acid lactones (CGLs); a significant correlation between CGLs and neuroprotective efficacy was observed (R^2 = 0.98). In conclusion, this study showed that roasted coffees are high in lipophilic antioxidants and CGLs, can protect neuronal cells against oxidative stress, and may do so by modulation of the ERK1/2 and JNK signaling pathways.

KEYWORDS: Coffee; antioxidant; neuroprotection; reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS) are oxygen derivatives produced during aerobic life processes (*1*). In humans, ROS activities are usually well controlled by antioxidant enzymes (e.g., superoxide dismutase and catalase) (*2*), thiol-containing peptides (e.g., glutathione) (*3*), metabolites (e.g., uric acid) (*4*), and dietary antioxidants (e.g., vitamin C, vitamin E, and phenolics) (*5*,*6*). Genetic defects of genes that encode antioxidant enzymes, insufficient consumption of dietary antioxidants, aging, and numerous other stress processes may lead to increased levels of ROS. Uncontrolled accumulation of ROS is detrimental to cells because of their undesired oxidative effects on biological molecules such as proteins, lipids, and DNA (*7*−*9*). The brain is particularly vulnerable to oxidative stress because of its high content of polyunsaturated fatty acids and low catalase activity (*10*). It has been well documented that ROS are involved in neurodegenerative processes, which may lead to diseases such as Alzheimer's and Parkinson's (*11*, *12*). The molecular mechanisms underlying oxidative stress-induced neuronal damage are emerging. Members of the mitogen-activated protein kinase (MAPK) family, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase 1 and 2 (JNK1/2), have been implicated with cellular degeneration and apoptosis of neurons subjected to oxidative stress (*13*).

Coffee is one of the most popular beverages around the world (*14*), constituting a significant portion of daily beverage intake in many Western countries (*15*−*17*). With the advancement of the research on antioxidants and health, coffee has been recognized as a rich source of dietary antioxidants with potential to improve health (*18*−*20*). Phenolics and melanoidins are the two main groups of antioxidants found in coffee brews (*19*, *21*). Green coffee beans are abundant in phenolics, especially the chlorogenic acids (i.e., caffeoylquinic acids (CQA), feruloylquinic acids (FQA), dicaffeoylquinic acids (diCQA), and, in smaller amounts, *p*-coumaroylquinic acids (*p*-CQA) and their derivates) (*22*). Melanoidins are formed during coffee roasting via the Maillard reaction, where the carbonyl groups of reducing sugars condense with the amino groups of amino acids or peptides (*21*, *23*).

Investigations of the effects of coffee on human brain functions have been focusing on caffeine because of its well known

*Corresponding authors. (Y.-F.C.) Phone: 847-646-3878. Fax: 847-646-3441. E-mail: yifang.chu@kraft.com. (I.H.C.) Phone: 886-2-28267905. Fax: 886-2-28273123. E-mail: hjcheng@ym.edu.tw.

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stimulating effects and its permeability across the blood−brain barrier (*24*). Recently, increasing attention has been directed to other coffee components, especially chlorogenic acid derivatives and metabolites because of their abundance in coffee (*25*−*27*). In light of the detrimental role of ROS in neurodegenerative processes (*11*, *12*) and coffee as a major dietary source of antioxidants (*18*−*20*), we are interested in whether antioxidants in coffee can protect the brain tissue against oxidative stress. Key to the biological relevancy of our question is the permeability of coffee antioxidants across the blood−brain barrier and their bioavailability in the brain tissue. Even though there is a lack of direct pharmacokinetic data, there is evidence that coffee components other than caffeine can reach the brain tissue, as exemplified by 3,4-diferuloyl-1,5-quinide (DIFEQ), a chlorogenic acid derivative (*27*). Further more, chlorogenic acids and caffeic acid, both present in coffee, have been shown to be neuron-protective in vivo under pathological conditions in animal models (*28*, *29*). This suggests their bioavailability in the brain tissue or at least suggests such a possibility under pathological conditions, for it has been shown that pathological conditions can compromise the blood−brain barrier (*30*). In addition, coffee antioxidants may at least be protective to brain tissue outside the blood−brain barrier, where they can be readily reached by well-established blood−brain barrier impermeable drugs (*31*).

The aim of this study was to investigate the potential benefits of coffee on neuronal cell survival against ROS. Decaffeination and roasting are two of the main processes in coffee manufacturing, and both processes markedly affect the chemical composition of coffee (*32*, *33*). To study the impact of decaffeination and roasting, coffee was selected from the following four groups: green decaffeinated (GD), green regular (GR), roast decaffeinated (RD), and roast regular (RR).

MATERIALS AND METHODS

Chemicals. Water (EMD, Gibbstown, NJ), acetonitrile, and methanol (Fisher Scientific, Boston, MA) were of HPLC-grade purity. AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] was purchased from Wako Chemicals USA (Richmond, VA). Fluorescein (sodium salt) (Fl) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Aldrich (Milwaukee, WI). Randomly methylated β-cyclodextrin (Trappsol, Pharm grade, RMCD) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL). Folin−Ciocalteu's phenol reagent, sodium carbonate, gallic acid, and 5-caffeoylquinic acid [1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)] were purchased from Sigma (St. Louis, MO). Formic acid of ACS reagent grade was obtained from EMD (Gibbstown, NJ).

Coffee Sample Preparation. Coffee beans (*Coffea arabica*) of Brazilian origin were subdivided into four groups: green roast (GR), green decaffeinated (GD), roast regular (RR), and roast decaffeinated (RD). Decaffeination was carried out using a supercritical carbon dioxide ($scCO_2$) extraction process. Briefly, green coffee beans were mixed with water to a moisture content of 50% and were then added to an extractor where liquid carbon dioxide was pumped in at an operating pressure of 300 atms and heated to 150 °C. Liquid carbon dioxide was recirculated between the extractor and a scrubber, where caffeine was removed from liquid carbon dioxide using water. Coffee beans were roasted to a medium roast color (12 La) in a Neotec RFB10 roaster with a batch size of 1.5 kg, a roast time of 255 s, and a moisture target of ∼4%. Coffee beans were ground using a Mahlkoenig Grinder to a medium grind size (∼450 μm) and brewed using standard drip filter preparation at 50 g coffee per liter of water. Coffee brews were freeze-dried using a mini freeze drier (Thermo Fisher Powerdry L3000). The caffeine contents of these freeze-dried coffee brews are 40.40 mg/g (GR), 1.60 mg/g (GD), 42.80 mg/g (RR), and 1.95 mg/g (RD), which were determined by a HPLC method at Brunswick laboratories (Norton, MA). These freeze-dried coffee samples were used for further chemical, biochemical, and cell culture characterization.

Oxygen Radical Absorbance Capacity (ORAC) Assays. To measure ORAC values in the hydrophilic fraction ($ORAC_{hydro}$), freeze-dried brewed coffee samples (5 g) were extracted with acetone/water (20 mL, 50:50 v/v) on an orbital shaker at room temperature for 1 h. The mixtures were centrifuged at 1972*g* in a ROTANTA 460R centrifuge (GMI, Ramsey, MN). The $ORAC_{hydro}$ values of the supernatants were measured, using a method adapted from Ou (*34*), on a FL600 plate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by the sKC4 3.0 software. The excitation wavelength was set at 485 (±20) nm and the emission wavelength set at 530 (±25) nm. To measure the ORAC values in the lipophilic fraction ($ORAC_{lipo}$), freeze-dried brewed coffee samples (5 g) were extracted with hexane/dichloromethane (10 mL, 50:50 v/v) twice. ORAC values of the combined organic phase were measured following previous published methodology (*35*, *36*) using the protocol above.

Primary Cortical Neuronal Culture. Primary neuronal cell cultures were generated from postnatal day 0 C57/BL6 mice. The cortex was removed in Hanks' balanced salt solution (Sigma, H4642) and dissociated by incubating for 30 min in papain (Sigma, P4762) at 37 °C. Dissociated cells were seeded onto poly-L-lysine-coated plastic plates (Corning) in minimal essential medium (Invitrogen, 11090081) and incubated at 37 °C and 5% CO_2 for 4 h. Cells were grown at 37 °C and 5% CO_2 in neural basal medium (Invitrogen, 21103049) supplemented with B-27 and L-glutamine. To reduce the proliferation of glial cells, 1 μM 5-fluoro-2′-deoxyuridine (FdU, Sigma, F0503) was added to the medium. After four days, the medium was replaced with neural basal medium without FdU and allowed to grow for an additional four days. All experiments were performed on cells incubated in vitro for eight days. Immunocytochemistry staining indicated that greater than 90% of the cultured cells were neurons.

Cell Survival. Cell survival was quantified using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay that measures mitochondrial succinate dehydrogenase conversion of MTT to a purple formazan (*37*, *38*). Cells were seeded into 96-well plates at 10^5 cells/well and grown at 37 °C and 5% CO_2 for 24 h before experiments. The cells were pretreated with media containing 0, 0.5, 5, or 50 ng/mL of different coffee extracts for 2 h. The media were then removed, and the cells were washed with fresh media with no coffee. Cells were then incubated in media containing H_2O_2 (500 μM) for 30 min at 37 °C and 5% CO_2. The cells were then replenished with media containing MTT (Genemark Taiwan, 0.5 mg/mL) and incubated at 37 °C for 4 h. After the media had been discarded, 100 μL of lysis buffer containing 10% SDS and 20 mM HCl was applied to each well to dissolve the formazan crystals, and the absorbance at 570 nm of each well was measured on a FL600 plate reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by KC4 3.0 software. Percent cell survival was calculated as $(OD570_{treated}/OD570_{nontreated}) \times 100\%$.

Immunocytochemistry. Cells (2.5×10^5) were plated on 12-mm^2 glass coverslips, treated with 50 ng/mL different coffees extracts for 2 h, rinsed with media, treated with 500 μM H_2O_2, and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at room temperature for 1 h. Coverslips were blocked for 1 h in a PBS-based blocking solution containing 0.03% Triton X-100 and 10% fetal bovine serum (Biological industries). All coverslips were incubated overnight at 4 °C with either the mouse monoclonal antibody against microtubule-associated protein 2 (MAP-2) (MAB378, Chemicon, USA) or the mouse polyclonal antibodies against glial fibrillary acidic protein (GFAP) (Z0334, DAKO, USA). Coverslips were rinsed with (cold) PBS and then incubated with the secondary antibody, either Texas-Red goat antimouse IgG (103007, AbD, USA) or FITC-conjugated goat antirabbit IgG (AP132F, Chemicon) in blocking solution for 1 h at room temperature. Omission of primary antibodies served as negative controls. Vectashield with DAPI (Vector Laboratories, Inc., USA) was applied to visualize nuclei. Fluorescent images of cells were captured by a CCD camera (DP50) mounted on an Olympus fluorescence microscope (BX-52, Olympus, Tokyo, Japan) equipped with a mercury arc lamp.

Western Immunoblot Analysis. Primary neurons (5×10^6 cells in a 6-well plate) were pretreated with 50 ng/mL of coffee for 2 h, rinsed, and then 500 μM H_2O_2 for 30 min. The cells were lysed in 1 mL of ice-cold RIPA buffer, containing 1% NP-40, 50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.25% Na-deoxycholate, protease inhibitor cocktail (Roche, 04693116001), and phosphatase inhibitor cocktail (Roche, 04906845001), and centrifuged at 1000*g* for 15 min. The supernatant was separated and stored at −20 °C.

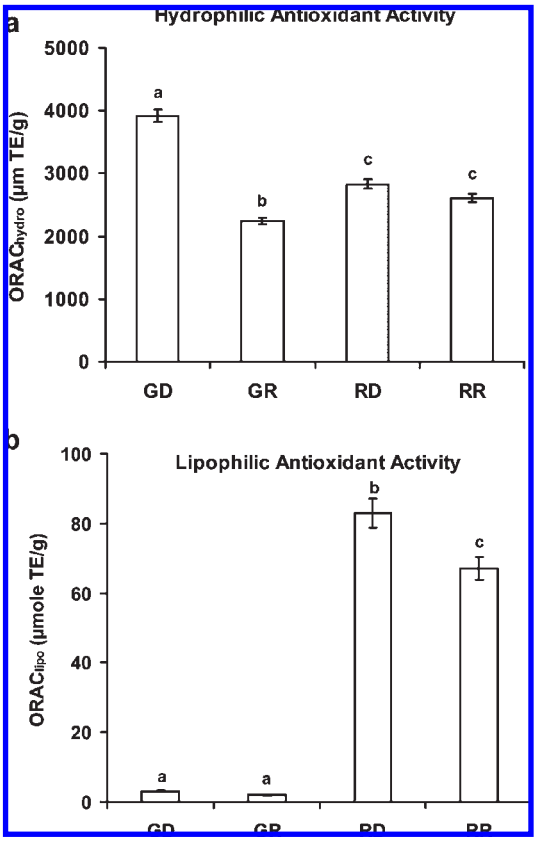

Figure 1. Oxygen radical absorbance capacity (ORAC). Hydrophilic (**a**) and lipophilic ORAC (**b**) of green decaffeinated (GD), green regular (GR), roasted decaffeinated (RD), and roasted regular (RR) coffee. Values are the mean of three determinations; error bars indicate the standard deviations; invisible error bars indicate that standard deviations are smaller than the markers used in the graphs. Statistical analysis was performed using one-way ANOVA followed by Newman−Keuls post-test. Significant differences exist between samples denoted by different letters; $P < 0.05$.

The protein concentration was determined using a Bradford protein assay kit (Strong Biotech, PAD500). Protein samples (66.5 mg) were heated at 95 °C for 5 min and analyzed by 10% SDS−PAGE. Western blotting was performed using (mouse) anti-ERK1/2 (Cell Signaling, 9102), (mouse) anti-JNK (Cell Signaling, 9252), (mouse) antiphospho-ERK1/2(Cell Signaling, 9101), and (mouse) antiphospho-JNK(Cell Signaling, 9251), and (mouse) anti-α-tubulin (Sigma, T5168) antibodies. Immunoblot signals were developed by chemiluminescent HRP substrate (Millipore). Multi Gauge v3.0 was used for image acquisition and data analysis.

Total Polyphenolic Contents. The total polyphenolic content of coffee samples was measured based on the method established by Singleton and Rossi (*39*). One milliliter of gallic acid standard or coffee sample extract was mixed with 15 mL of water and 1.0 mL of Folin−Ciocalteu reagent, and incubated at room temperature for 10 min. After the addition of 3.0 mL of a 20% sodium carbonate and incubation at 40 °C for 20 min, the absorbance was measured at 755 nm using an Agilent 8453 UV−visible spectrophotometer (Waldbronn, Germany). Total polyphenolic contents were expressed in milligrams of gallic acid equivalents per gram of coffee powder.

Quantitative Analysis of Chlorogenic Acids and Lactones. Coffee samples (0.1 g) were extracted with 12 mL of methanol in 50 mL-conical tubes. Mixtures were sonicated at room temperature for 10 min, followed by shaking at 300 rpm for 30 min. After centrifugation at 1972*g* in a ROTANTA 460R centrifuge, supernatants were analyzed with a Shimadzu HPLC system (pump, LC-20AT; auto sampler, SIL-HTC; UV detector, SPD-20A) equipped with a 4.6 × 250 mm Symmetry ShieldTM-C18 (5 μm) column (Waters, Milford, MA). The mobile phase consisted of H_2O containing 0.4% formic acid (A) and acetonitrile (B). Separation was carried out at room temperature at a flow rate of 0.8 mL/min. The percentage of B in the mobile phase was increased from 0 to 50% over 40 min, then increased to 95% over 5 min and maintained at 95% for a further 5 min. The percentage of B in the mobile phase was then decreased to 0% in 3 min, and maintained at 0% for 5 min for column re-equilibration. A SCIEX 4000 Q-trap detector with ESI source at positive mode was used to monitor ions at *m*/*z* 355, 369, 321, 337, 351, 499, and 517. Identification of caffeoylquinic acids (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs), and their lactone derivatives was achieved by comparison of the MS data with the literature values provided by Farah et al. (*33*, *40*). Quantification of specific compounds separated by HPLC was based on UV detection at 325 nm using 5-caffeyolquinic as the reference (*41*). The acquired data was analyzed using Analyst software (v1.4.1).

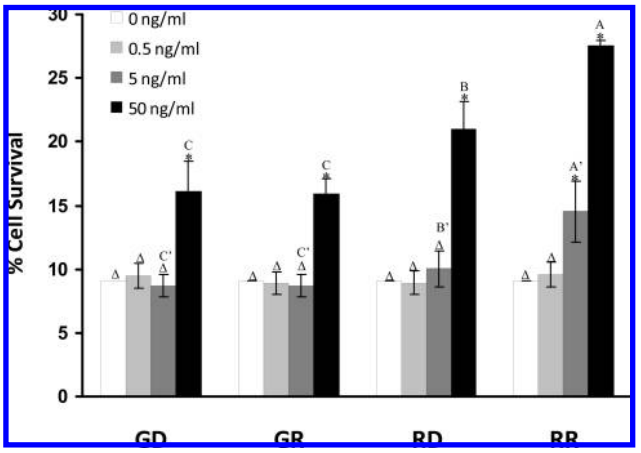

Figure 2. Coffee extracts protect primary neuron cells against H_2O_2. Values are the means from three independent experiments, each replicated in triplicate. Statistical analysis was performed using two-way ANOVA. Within a type, different symbols represent significant difference; $p < 0.05$. Between coffee types, but within a specific dose, different letters represent significant differences; $p < 0.05$.

RESULTS AND DISCUSSION

Antioxidant Activities of Coffees. The ORAC assay is a well-established and widely used method to characterize antioxidant activities of foods and beverages (*34*, *42*). Antioxidant activities were measured on the hydrophilic and lipophilic fractions of green regular (GR), green decaffeinated (GD), roast regular (RR), and roast decaffeinated (RD) *Brazilian arabica* coffee beans (*34*) (**Figure 1**). Comparing the ORAC$_{hydro}$ values of decaffeinated coffees with those of the regular coffees (GD vs GR or RD vs RR), we observed that the decaffeinated coffees had elevated hydrophilic antioxidant levels (**Figure 1a**). This result indicates that caffeine has little or no direct antioxidant activity and is consistent with a previous report (*43*). Examination of the ORAC$_{lipo}$ values of GD vs RD or GR vs. RR show that roasting led to an approximately 30-fold increase in the antioxidant activities of the coffees in the lipophilic fractions (**Figure 1b**).

Protection of Neuron Cells from H_2O_2. In vitro chemical antioxidant activity as measured by ORAC does not necessarily correlate with beneficial biological effects (*20*, *44*). To evaluate the biochemical impact of the antioxidant activity associated with coffee, these coffee samples were evaluated for their potential to protect primary cortical neuron cells from oxidative stress.

The physiological concentration of H_2O_2 is at the micromolar level (*45*, *46*). Elevated levels of H_2O_2 (up to several hundred micromolars) have been used in cell culture models to study H_2O_2 cytotoxicity (*46*, *47*). In this study, the concentration of H_2O_2 was 500 μM. The optimal concentration of coffee to protect H_2O_2-induced cell death in neurons was determined. Primary neuron cell cultures were pretreated with media containing 0, 0.5, 5, or 50 ng/mL

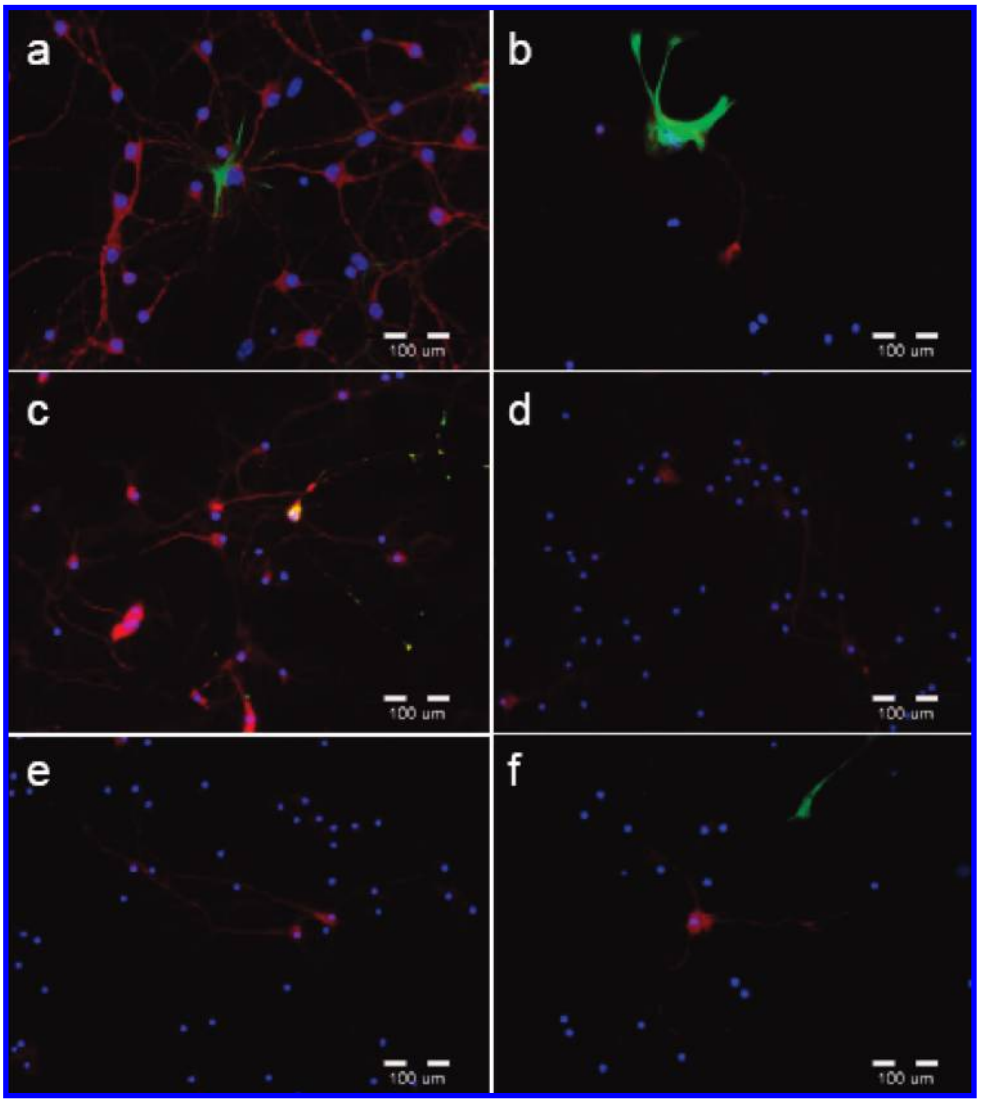

Figure 3. Morphology of primary neuron cells after treatment of H_2O_2 with or without coffee pretreatment. Primary neuron cell cultures were pretreated with 50 ng/mL of coffee extracts for 2 h and then incubated with a 500 μM H_2O_2 for 30 min. (**a**) Control, (**b**) H_2O_2 only, (**c**) RR + H_2O_2, (**d**) RD + H_2O_2 (**e**) GR + H_2O_2, and (**f**) GD + H_2O_2. Blue, nucleus; green, astrocyte; red, neuron.

of coffee extracts for 2 h, washed with media, and were then incubated with H_2O_2 (500 μM) for 30 min. Media containing H_2O_2 was removed, cells were rinsed, and cell survival was quantified by the MTT assay using untreated cells as controls. We kept the concentration of coffee samples below 50 ng/mL because at higher concentrations, the dark brown color of the coffee sample interfered with the MTT assay. In our pilot experiments, we varied the incubation times (0−4 h) of coffee extracts with neuron cells and found that the cell survival at 4 h was similar to that at 2 h. We selected an incubation time of 2 h because it was an appropriate time window not only for observing cell survival but also for subsequent cell signaling study. No significant protection was observed until the concentration of the coffee extract reached 50 ng/mL (**Figure 2**), indicating that, at the highest concentration tested (50 ng/mL), the overall antioxidant activity of the coffee extracts already exceeded a threshold in contributing to neuron protection. Nardini et al. reported that 1 h after the ingestion of 200 mL of coffee brew (60 g of coffee brewed with 1 L of water), human plasma concentration of coffee phenolics reached about 90 ng/mL (*48*), which is much higher than the concentration of coffee extracts (50 ng/mL) used in our study, suggesting that our dosage might be physically achievable at least on the plasma level (data are not available indicating physiologic concentrations in brain tissue).

Protection of neurons against H_2O_2 damage was observed for all tested coffees (**Figure 2**). GD and GR exhibited comparable degrees of protection (**Figure 2**) despite their different $ORAC_{hydro}$ values (**Figure 1**). These results suggested that, at the highest concentration tested (50 ng/mL), overall antioxidant activity of the GR was sufficient to provide protection to neuronal cells, almost doubling the percent of surviving cells. Roasted coffees appeared to have better protective effects than those of the green coffees (**Figure 2**). A major difference between the roasted (RD and RR) and green coffee (GD and GR) is that the roasted coffee had higher $ORAC_{lipo}$ values (**Figure 1b**). Lipophilic antioxidants should be more effective in a lipid-rich environment, as shown by Daglia et al. (*16*) who reported that the lipophilic fraction of roasted coffee extracts demonstrated potent inhibition of lipid peroxidation in an ex vivo rat liver cell microsome system. The brain is a lipid-rich tissue, with ~8% lipid content on a wet weight basis (*49*). Therefore, these results suggest that the lipophilic antioxidant activity may have accounted for elevated neuronal protection against H_2O_2 with roasted coffees.

Immunocytochemistry was used to further observe cellular morphology after coffee treatments (**Figure 3**). Primary cortical cell cultures were pretreated with 50 ng/mL of coffee for 2 h, rinsed with incubation medium, and then incubated with 500 μM

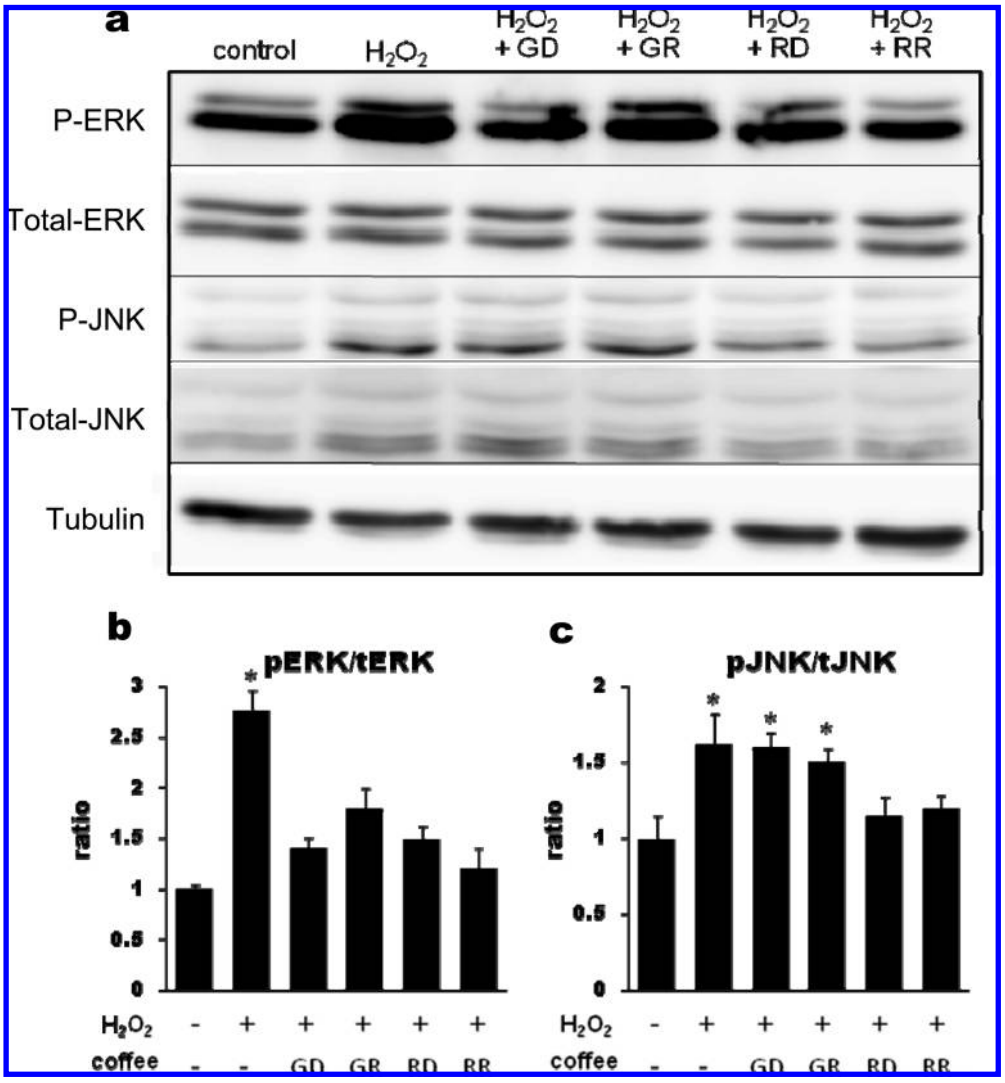

Figure 4. Activation of signaling kinases in the presence of coffee. (**a**) Neuron cells treated with coffee extracts and H_2O_2 were analyzed for ERK1/2 (44/42 kDa) and JNK1/2 (46/54 kDa) activation by Western blots using phosphorylated-ERK and phosphorylated-JNK specific antibodies as indicated in Materials and Methods. The same blot was subsequently analyzed using antibodies that recognize total ERK or total JNK and tubulin to confirm equal loading on each lane. (**b**,**c**) Ratio of phosphorylated-ERK/total ERK (**b**) and phosphorylated-JNK/total JNK (**c**) were represented as the mean ± SD from four independent experiments. Data are presented as the percentage of the basal level of phosphorylation (control = 1). *$p < 0.05$ vs control.

H_2O_2 for 30 min (no rinse before fixation). After fixation, they were immunolabeled with cellular specific markers for neurons (MAP2, red) and astrocytes (GFAP, green) and stained with a nuclear dye (DAPI, blue). **Figure 3a** shows the normal morphology of neuronal cells, which occupied greater than 90% of the cell population. Visual examination of immunocytochemical staining demonstrated that the MAP2 immunoreactive profile was clearly diminished and even the nuclear staining became weaker, after exposure to H_2O_2 (**Figure 3b**). Pretreating the culture with different types of coffee partially reduced the H_2O_2 damage, but surviving neurons showed shorter and thinner staining of axons and dendrites (**Figure 3c**−**f**). Cultures treated with roasted coffee (**Figure 3c**,**d**) had more MAP2 positive neurons than cultures treated with green coffee (**Figure 3e**,**f**), confirming our previous cell survival results.

There is growing evidence that ROS-induced cell death, such as apoptosis, is executed through cell signaling cascades (*50*). It has been suggested that members of the mitogen-activated protein kinase (MAPK) family may play a critical role in neuronal apoptosis (*37*, *38*). Some investigations suggested that activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) plays a role in neuronal apoptosis, but there is also evidence to the contrary. In neuronal cells, phosphorylation of ERK1/2 is primarily induced by growth factors and is involved in cellular proliferation, differentiation, and development, as well as apoptosis (*37*, *38*). There is more conclusive evidence indicating that activation of c-Jun N-terminal kinase (JNK) plays a significant role in neuronal apoptosis. Phosphorylation of JNK is preferentially induced by environmental stresses and inflammatory cytokines, and has been shown to promote neuronal cell death (*51*, *52*).

We hypothesized that the protective effects of coffee against H_2O_2-induced neuron cell death may be mediated through the inhibiting activation of ERK1/2 and JNK1/2. We tested our hypothesis by monitoring the phosphorylation status of ERK1/2 and JNK1/2 using antibodies against ERK1/2 and phosphorylated ERK1/2, and antibodies against JNK and phosphorylated JNK1/2, respectively. Our results indicated that a 30-min treatment of neurons with H_2O_2 (500 μM) increased the phosphorylation of ERK1/2 and JNK1/2 (**Figure 4a**). Pretreatment of the neurons with all coffee lowered the level of phosphorylated ERK (**Figure 4b**). However, only roasted coffee lowered the level of

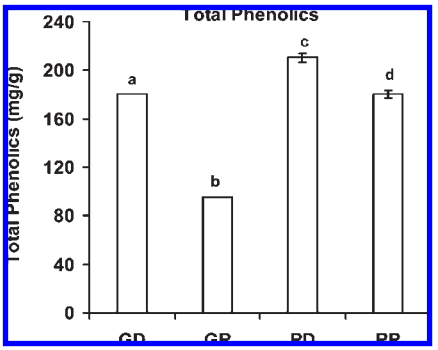

Figure 5. Total phenolic contents in different coffees measured by the Folin−Ciocalteu assay. Values are the mean of three determinations; error bars indicate the standard deviations. Invisible error bars indicate that standard deviations are smaller than the markers used in the graphs. Statistical analysis was performed using one-way ANOVA followed by Newman−Keuls post-test. Significant differences exist between samples denoted by different letters; $P < 0.05$.

phosphorylated JNK (**Figure 4c**). These results are in agreement with our findings that roasted coffees have better protective effects against H_2O_2-induced cell death (**Figures 2** and **3**). Moreover, these results indicate that the protective effects of coffee against H_2O_2-induced neuron cell death may be mediated by inhibiting activation of ERK1/2 and JNK1/2 signaling pathways. Cho et al. has recently shown in the PC12 cell line that instant coffee (1000−5000 ng/mL) and 5-*O*-caffeoylquinic acid (360−1800 ng/mL) can inhibit H_2O_2-induced cell death via inhibiting the phosphorylation of JNK and p38 mitogen-activated protein kinase (MAPK) (*53*). As a majority of coffee consumption is roast and ground (R&G) coffee, it should also be noted that the chemical compositions vary distinctly between instant and R&G coffee. The difference is mostly attributed to the extensive water evaporation heating stage that is involved in preparing instant coffee. The primary cell system employed in our study is of physiological sophistication and fidelity. Both differences, i.e., coffee samples and cell system, may explain the better response (>20-fold) of neuronal survival to coffee extract observed in our study compared to those observed in the immortalized PC12 cell lines.

Profile of Phenolics in Coffees. In vitro and neuron cell culture data indicate that coffees prepared by different processing procedures have different antioxidant activities (**Figures 1**, **2**, and **3**). This is likely a manifestation of the difference in chemical compositions of the coffees due to the complex physical and chemical changes that occur during the decaffeination and roasting processes (*32*, *33*). It has been postulated that characterizing the chemical profiles of these coffees may help reveal potential neuron protective agents in coffees.

Phenolic compounds and melanoidins are the two main chemical groups contributing to the antioxidant activity in coffee. The focus of the study was to characterize and quantitate the phenolic compounds in brewed coffee because (1) they are present both in green and roasted coffees (*33*, *54*); (2) roasting affects their profiles (*33*); and (3) analytical methods for the identification of phenolic compounds are well established and readily available (*40*, *41*). Characterization and quantification of melanoidins were not carried out in this study because (1) they are not present in green coffees; (2) low molecular weight melanoidins are negatively charged (*55*) and would not likely diffuse into cells through the negatively charged cell membrane; (3) high molecular weight melanoidins mainly exert their biological function extracellularly (*56*); and (4) the exact structures of melanoidins are complex and yet to be elucidated (*55*, *57*).

Table 1. Profile of Chlorogenic Acids (CGA) and Chlorogenic Acid Lactones (CGL)[a]

	GD	GR	RD	RR
3-CQA	72.00	22.24	15.74	30.34
4-CQA	74.94	31.84	17.55	43.28
5-CQA	118.69	150.31	23.67	65.48
3-FQA	ND	ND	ND	1.30
4-FQA	5.54	1.98	ND	2.96
5-FQA	11.80	11.80	6.21	12.07
3,4 diCQA	11.89	8.92	3.30	5.42
3,5 diCQA	9.29	10.48	2.99	1.88
4,5 diCQA	11.14	9.73	2.99	2.96
total CGA (g/kg)	**315.29**	**248.90**	**72.45**	**165.69**
3-CQL	ND	ND	3.71	ND
4-CQL	ND	ND	ND	11.34
5-CQL	ND	ND	3.71	10.87
3-FQL	ND	ND	ND	1.06
3-FQL	ND	ND	ND	ND
5-FQL	ND	ND	ND	ND
total CGL (g/kg)	**0.00**	**0.00**	**6.86**	**23.27**

[a] CQA, caffeoylquinic acid; FQA, feruloylquinic acid; diCQA, dicaffeoylquinic acid; CGA, chlorogenic acids; CQL, caffeoylquinic acid lactone; FQL, feruloylquinic acid lactone; CGL, chlorogenic acid lactone; ND, not detected.

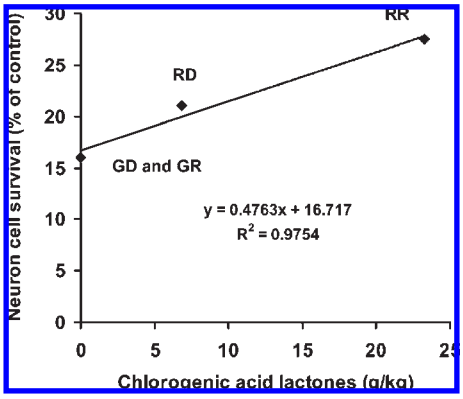

Figure 6. Correlation between neuron cell survival and contents of chlorogenic acid lactones in different coffees. Data of neuron cell survival (**Figure 3**) and data of contents of chlorogenic acid lactones (**Table 1**) were plotted together to show the correlation.

The total phenolic content of brewed coffee was determined using the Folin−Ciocalteu assay (**Figure 5**). Specific phenolic compounds were identified and quantified using LC-MS/UV and include isomers of caffeoylquinic acids (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs), and their lactone derivatives (**Table 1**). GD has been found to contain more total phenolic content on a per gram basis compared to GR (**Figure 5**). The same trend is seen when RD is compared with RR (**Figure 5**). Roasted coffees contain less chlorogenic acids but more chlorogenic acid lactones than the green coffees (**Table 1** and **Figure 6**). These results are consistent with a previous report that chlorogenic acids are lost during roasting either by incorporating into melanoidins (*57*) or by converting to lactones (*33*). Surprisingly, the roasted coffee seemed to show higher phenolic content than green coffee in the Folin−Ciocalteu assay (**Figure 5**), which was contradictory to the phenolic profiling data (**Table 1** and **Figure 6**). This apparent contradiction is likely due to the presence of melanoidins in the samples of the roasted coffee extracts, which, unfortunately, positively interfere with the Folin−Ciocalteu assay (*21*, *57*), leading to an overestimation of total phenolics.

Concentrations of coffee bioactives in the brain are important determinants of whether the protective effect observed is

biologically relevant. Monterio et al. reported that the plasma level of chlorogenic acids can reach 3.3 mg/mL in 2 h after ingestion of 190 mL of brewed coffee roasted to a light medium degree (*58*). So far, there is no direct report as to whether chlorogenic acids can pass through the blood−brain barrier. However, the blood−brain barrier has been found to be permeable to epigallocatechin gallate (EGCG, MW 458), the bioactive phenolics in green tea (*59*). The molecular weights of chlorogenic acids and their lactones are in the range of 354−516. Given the high plasma levels of chlorogenic acids after coffee ingestion and the similar molecular weights, chlorogenic acids are likely to permeate the blood−brain barrier. It has been found in a mouse model that chlorogenic acid affects spontaneous locomotor activity, suggesting that chlorogenic acid or its metabolite could pass the blood−brain barrier (*26*). The chlorogenic acid lactones are less polar than their parent compounds and should be more permeable to the blood−brain barrier. Interestingly, previous studies have shown that there are specific binding sites for chlorogenic acid lactones in brain cells, including the adenosine transporter (*27*). Our results show a significant correlation ($P < 0.05$) between the levels of chlorogenic acid lactones in coffee (**Figure 6**) and neuron cell survival (**Figure 2**). This suggests that chlorogenic lactones might contribute to the increased protective effects against H_2O_2-induced death of neuron cells. It is thus suggested that the protective effects of pure chlorogenic acid and chlorogenic acid lactones should be investigated in future studies.

In this study, the antioxidant levels (hydrophilic vs lipophilic) and neuroprotective effects of coffee prepared from different bean processing procedures were characterized. It was found that while all brewed coffee tested were high in hydrophilic antioxidant activity (> 2000 μmol TE/g), roasted coffee extracts had a dramatic, approximately 30-fold, increase of lipophilic antioxidant activity compared to that of green coffees. Preincubation with all brewed coffee increased primary neuron survival under oxidative stress conditions, with the roasted coffee showing stronger protective effects. The brain is a lipid-rich organ (*49*), where lipophilic antioxidants may act more efficiently. Accordingly, the elevated lipophilic antioxidant activity in the roasted coffee may account for the observed higher neuronal protection against oxidative stress. Mitogen activated protein kinases, extracellular signal-regulated kinases 1 and 2, and c-jun N-terminal kinase have been reported to play pivotal roles in the regulation of stress-induced neuronal cell death. All coffee tested in this study inhibited the H_2O_2-induced ERK1/2 activation, indicating a potential attenuating effect in apoptosis. Interestingly, only roasted coffee extracts inhibited JNK activation, evidencing a distinctive neuroprotective benefit. Analysis of coffee phenolic compounds in coffee extracts revealed a significant correlation between levels of coffee CGLs and protection of neuron cells against H_2O_2-induced cell death. Further investigations are warranted to identify key neuroprotective coffee bioactives and to elucidate their mechanisms of action in affording the neuroprotective activities observed in this study.

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