Mutation Research 661 (2009) 18–24

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Attenuation of oxidative neuronal cell death by coffee phenolic phytochemicals

Eun Sun Cho[a,1], Young Jin Jang[a,1], Mun Kyung Hwang[a,b], Nam Joo Kang[a,b], Ki Won Lee[b,*], Hyong Joo Lee[a,**]

[a] Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea
[b] Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea

ARTICLE INFO

Article history:
Received 2 May 2008
Received in revised form 22 October 2008
Accepted 24 October 2008
Available online 5 November 2008

Keywords:
Bcl-X_L
Chlorogenic acid
Instant decaffeinated coffee
Oxidative stress
Mitogen-activated protein kinases

ABSTRACT

Neurodegenerative disorders such as Alzheimer's disease (AD) are strongly associated with oxidative stress, which is induced by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2). Recent studies suggest that moderate coffee consumption may reduce the risk of neurodegenerative diseases such as AD, but the molecular mechanisms underlying this effect remain to be clarified. In this study, we investigated the protective effects of chlorogenic acid (5-*O*-caffeoylquinic acid; CGA), a major phenolic phytochemical found in instant decaffeinated coffee (IDC), and IDC against oxidative PC12 neuronal cell death. IDC (1 and 5 μg/ml) or CGA (1 and 5 μM) attenuated H_2O_2-induced PC12 cell death. H_2O_2-induced nuclear condensation and DNA fragmentation were strongly inhibited by pretreatment with IDC or CGA. Pretreatment with IDC or CGA also inhibited the H_2O_2-induced cleavage of poly(ADP-ribose) polymerase (PARP), and downregulation of Bcl-X_L and caspase-3. The accumulation of intracellular ROS in H_2O_2-treated PC12 cells was dose-dependently diminished by IDC or CGA. The activation of c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) by H_2O_2 in PC12 cells was also inhibited by IDC or CGA. Collectively, these results indicate that IDC and CGA protect PC12 cells from H_2O_2-induced apoptosis by blocking the accumulation of intracellular ROS and the activation of MAPKs.

1. Introduction

Alzheimer's disease (AD), a common type of dementia, is a progressive neurodegenerative disorder characterized by the accumulation of senile plaques containing amyloid β (Aβ) and neurofibrillary tangles composed of phosphorylated tau in the brain. The cause of AD is uncertain, but several recent studies have implicated reactive oxygen species (ROS)-induced oxidative stress in its pathogenesis [1,2]. Hydrogen peroxide (H_2O_2) is a major mediator of oxidative stress [3], and Aβ plays a critical role in the development and progression of AD via the generation of ROS such as H_2O_2 [4]. H_2O_2 in turn is involved in the production of highly reactive hydroxyl radicals via Fenton's reaction, which promotes apoptosis [5]. Both these by-products and H_2O_2 itself can react with nearly all cellular macromolecules to damage proteins, lipids, mitochondria, and DNA [6].

H_2O_2-induced apoptosis is accompanied by changes in apoptosis-related factors such as the Bcl-2 family of regulatory proteins. Among its members, Bcl-X_L is a major antiapoptotic protein [7] that reportedly protects neurons against H_2O_2-induced cell death [8]. The cysteine protease caspase-3 is a key executor of apoptosis that is also reportedly activated by H_2O_2 [9]. The cleavage of poly(ADP-ribose) polymerase (PARP) by activated caspase-3 is a hallmark of apoptosis [10]. In addition, mitogen-activated protein kinase (MAPK) signaling is involved in cellular events such as gene expression, mitosis, and apoptosis via the phosphorylation of target proteins at specific serine and/or threonine residues. In particular, the activation of c-Jun N-terminal protein kinase (JNK) and p38 MAPK plays a critical role in the induction of apoptosis in neurons [11,12].

Coffee, as a rich source of caffeine, has been reported to have an effect on neurodegenerative disorders such as Parkinson's disease [13]. Similarly, a recent epidemiologic study reported that moderate

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β; CGA, chlorogenic acid; DAPI, 4,6-diamidino-2-phenylindole; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; H_2O_2, hydrogen peroxide; IDC, instant decaffeinated coffee; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PC12, PC12 rat pheochromocytoma; ROS, reactive oxygen species.

* Corresponding author at: Department of Bioscience and Biothechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea. Tel.: +82 2 2049 6178; fax: +82 2 3436 6178.
** Corresponding author at: Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, San 56-1, Shillim-dong, Gwanak-gu, Seoul 151-921, Republic of Korea. Tel.: +82 2 880 4853; fax: +82 2 873 5095.
E-mail addresses: kiwon@konkuk.ac.kr (K.W. Lee), leehyjo@snu.ac.kr (H.J. Lee).
[1] These authors contributed equally to this work.

0027-5107/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2008.10.021

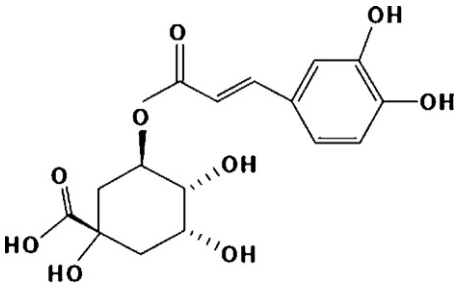

Fig. 1. Chemical structure of CGA.

daily consumption of caffeine was significantly associated with a reduced risk of AD [14]. In an AD murine model, caffeine was shown to have an effect on cognitive protection through the suppression of Aβ production; specifically, caffeine was found to antagonize the activity of the adenosine A receptor [15,16].

Although caffeine is the only ingredient believed to have major neuroprotective effects, coffee is a major dietary source of phenolic compounds [17]. Chlorogenic acid (5-*O*-caffeoylquinic acid; CGA) (Fig. 1) which is formed by the esterification of quinic acid with *trans*-cinnamic acid [18] is a major phenolic compound in coffee; in fact, the CGA content of a 200 ml cup of coffee is 70–350 mg [17,19]. However, the mechanism underlying the neuroprotective effects of coffee and CGA against oxidative neuronal cell death and injury remains to be clarified. The PC12 rat pheochromocytoma (PC12) cell line is a useful model system for investigating neuronal cell death [20]. Several previous studies have shown that H_2O_2 triggers apoptosis in PC12 cells [3,21]. The present study investigated the potential protective effects of instant decaffeinated coffee (IDC) and CGA against H_2O_2-induced apoptosis in PC12 cells.

2. Materials and methods

2.1. Chemical

H_2O_2 was purchased from Junsei Chemical (Tokyo, Japan). CGA, 4,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Folin and Ciocalteu's phenol reagent, and trypan blue (0.4% solution) were purchased from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, horse serum, and a penicillin/streptomycin mixture were obtained from Gibco–BRL (Grand Island, NY, USA). Anti-PARP, anti-caspase-3, anti-JNK, and anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin antibodies were purchased from Sigma Chemical, while anti-Bcl-X_L, and anti-phosphorylated-JNK antibodies were obtained from Cell Signaling (Beverly, MA, USA). Anti-phosphorylated-p38 MAPK antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). All other chemicals were of analytical grade.

2.2. Sample preparation and measurement of the total coffee phenolic content

Distilled water (80 °C, 100 ml) was added to 10 g of a commercially prepared IDC powder and stirred for 5 min. The solution was then filtered through a membrane filter under a vacuum. The total phenolic content of the liquid was measured independently six times using the Folin–Ciocalteu method. Folin and Ciocalteu's phenol reagent (5 μl) was added to 50 μl of diluted sample (IDC) or standard solution (CGA) and shaken for 6 min, and then 50 μl of 7% Na_2CO_3 was added to the reaction mixture. The mixture was immediately diluted with 100 μl of distilled water and incubated for 90 min at 23 °C. After incubation, the absorbance at 750 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The total phenolic content in the IDC was expressed as milligrams per gram of CGA equivalents. A stock solution of 100 mg/ml IDC was used in this study.

2.3. Cell culture

PC12 cells, which were kindly supplied by Dr. Y.-J. Surh (Seoul National University, Seoul, Korea), were grown in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 0.1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air.

2.4. MTT assay

MTT is metabolized to an insoluble purple formazan by mitochondrial dehydrogenases, which are active only in live cells. Thus, cell viability was measured based on the formation of a purple formazan metabolite, which was solubilized by the addition of dimethyl sulfoxide (DMSO). PC12 cells (2×10^4 cells/well in 96-well plates) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with IDC or CGA, and then treated with 1 mg/ml MTT (final concentration) for 2 h. The dark blue formazan crystals in the intact cells were dissolved in DMSO and the absorbance at 570 nm was measured with a microplate reader. The results are expressed as the percent reduction in absorbance relative to that in the control cells.

2.5. Trypan blue exclusion assay

Trypan blue can be used to identify non-viable cells since it specifically interacts with damaged cell membranes. PC12 cells (10^5 cells/well in 6-well plates) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with IDC or CGA. After centrifugation at 600 × *g* for 6 min, the cells were resuspended in 200 μl of phosphate-buffered saline (PBS). The entire suspension was then mixed with 200 μl of 0.4% trypan blue solution and incubated for 5 min at room temperature. The cells were then loaded into a hemocytometer, and those exhibiting dye uptake were counted under a microscope (Olympus, Tokyo, Japan). The percentage of stained cells was based on 150 cells.

2.6. DAPI staining

The fluorescent dye DAPI was used to detect nuclear fragmentation, which is characteristic of apoptotic cells. PC12 cells (2×10^4 cells/well in 24-well plates) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with IDC or CGA, and then washed with PBS and fixed with 70% ethanol for 20 min. The fixed cells were then washed with PBS and stained with 1 μg/ml DAPI. Following 10 min of incubation, the cells were again washed with PBS, and the plates were observed under a fluorescence microscope (Olympus). The degree of nuclear fragmentation was evaluated based on the percentage of DAPI-stained cells from among 100–120 randomly chosen cells.

2.7. DNA fragmentation analysis

Apoptotic cells exhibit unique ladders of nucleotide fragments during agarose gel electrophoresis. PC12 cells (1.6×10^6 cells/8 ml in a 8.5-cm dish) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with IDC or CGA, and then washed and collected with ice-cold PBS and centrifuged at 200 × *g* for 10 min. Cellular DNA was isolated using a DNA isolation buffer [10 mM EDTA, 50 mM Tris–HCl (pH 8.0), 0.5% SDS, and 0.5 mg/ml proteinase K] and incubated for 4 h at 50 °C. After centrifugation at 10,000 × *g* for 15 min, the supernatants were extracted with an equal volume of phenol, chloroform, and isoamyl alcohol. The DNA was then mixed with 4 M NaCl and 100% ethanol and stored at −70 °C overnight. Each sample was then loaded onto a 1.8% Tris–boric acid–EDTA agarose gel and electrophoresed at 100 V for 30 min.

2.8. Measurement of intracellular ROS accumulation

We measured the intracellular production of ROS using a DCFH-DA assay. Dichlorofluorescin diacetate (DCFH-DA) is deacetylated in cells, whereupon its reaction with intracellular radicals (mainly H_2O_2) converts it into a fluorescent product, DCF, which is retained within the cells. PC12 cells (5×10^4 cells/well in 24-well plates) were preincubated in PBS containing 50 μM DCFH-DA for 20 min and then rinsed with PBS. The cells were then incubated at 37 °C with 200 μM H_2O_2 for 15 min with or without pretreatment with IDC or CGA, and then lysed (0.5% Triton X-100, 0.1% CHAPS, and 0.1 mM EDTA) and examined with a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan) with excitation at 488 nm and emission at 530 nm.

2.9. Western blot analysis

PC12 cells (2×10^5 cells/4 ml in a 6-cm dish) were incubated at 37 °C with 200 μM H_2O_2 for 24 h with or without pretreatment with IDC or CGA, washed and collected with ice-cold PBS, and then centrifuged at 600 × *g* for 10 min. The cell pellet was then resuspended in 100 μl of ice-cold lysis buffer (cell signaling) and incubated on ice for 30 min. After centrifugation at 1000 × *g* for 15 min, the supernatant was separated and stored at −70 °C. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). The proteins were then separated by SDS–PAGE and transferred onto a polyvinylidene difluoride transfer membrane,