Toxicology in Vitro 24 (2010) 375–381

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Protective effects of dieckol isolated from *Ecklonia cava* against high glucose-induced oxidative stress in human umbilical vein endothelial cells

Seung-Hong Lee [a], Ji-Sook Han [b], Soo-Jin Heo [c], Ji-Young Hwang [b], You-Jin Jeon [a,d,*]

[a] Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea
[b] Department of Food Science and Nutrition, Pusan National University, Busan 609-735, Republic of Korea
[c] Marine Living Resources Research Department, Korea Ocean Research and Development Institute, Ansan 426-774, Republic of Korea
[d] Marine and Environmental Research Institute, Jeju National University, Jeju 695-814, Republic of Korea

ARTICLE INFO

Article history:
Received 16 July 2009
Accepted 2 November 2009
Available online 5 November 2009

Keywords:
Ecklonia cava
Dieckol
High glucose
Oxidative stress
Protective effects

ABSTRACT

The effect of dieckol, one of phlorotannin polyphenol compound purified from *Ecklonia cava* (*E. cava*) against high glucose-induced oxidative stress was investigated using human umbilical vein endothelial cells (HUVECs), which is susceptible to oxidative stress. High glucose (30 mM) treatment induced HUVECs cell death, but dieckol, at concentration 10 or 50 μg/ml, significantly inhibited the high glucose-induced cytotoxicity. Furthermore, treatment with dieckol dose-dependently decreased thiobarbituric acid reactive substances (TBARS), intracellular reactive oxygen species (ROS) generation and nitric oxide level increased by high glucose. In addition, high glucose levels induced the overexpressions of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and nuclear factor-kappa B (NF-kB) proteins in HUVECs, but dieckol treatment reduced the overexpressions of these proteins. These findings indicate that dieckol is a potential therapeutic agent that will reduce the damage caused by hyperglycemia-induced oxidative stress associated with diabetes.

1. Introduction

Free radicals or reactive oxygen species (ROS) create oxidative stress, which leads to a variety of pathological lesions and often results in metabolic impairments such as inflammation, aging, cancer, and hypertension (Ames, 1998; Halliwell et al., 1992). Several recent studies have demonstrated that hyperglycemia can cause glucose to undergo autooxidation to generate intermediates that lead to the formation of ROS, nitric oxide (NO), peroxynitrite ($ONOO^-$), and advanced glycation end products (AGE), which cause various complications of diabetes such as nephropathy, retinopathy, and neuropathy. Moreover, hyperglycemia can reduce antioxidant enzyme defenses, thereby allowing ROS to accumulate, resulting in cellular and tissue damage (Giugliano et al., 1996; Baynes and Thorpe, 1999). Antioxidants can prevented pathological damage caused by hyperglycemia-induced oxidative stress associated with diabetes (Yokozawa et al., 2007a,b).

The brown alga *Ecklonia cava* is plentifully produced on Jeju Island in Korea. It is popular in Korea and Japan as a food ingredients, supplement of animal feed and fertilizers, and as a medicine. The total polyphenolic compounds (phlorotannins) in *E. cava* are richer than in other brown algae (Heo et al., 2003, 2005). Phlorotannin components of *E. cava* include phenolic secondary metabolites such as eckol (a closed-chain trimer of phloroglucinol), 6,6′-bieckol (a hexamer), dieckol (a hexamer), phlorofucofuroeckol (a pentamer) and triphlorethol-A that are influential for biological activities (Ahn et al., 2007; Kang et al., 2005a,b). Among these phlorotannins, dieckol is one of the major and active compounds. Its attributes include antioxidant activity (Ahn et al., 2007), antiallergic activity (Le et al., 2008), inhibition of human immunodeficiency virus-1 reverse transcriptase (Ahn et al., 2004), and inhibition of the expression of matrix metalloproteinase-1 (MMP-1) (Joe et al., 2006).

In the present study, we investigated the protective effects of dieckol isolated from *E. cava* against high glucose-induced oxidative stress using human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Materials

The brown alga *Ecklonia cava* (*E. cava*) was collected from the coast of Jeju Island, south Korea. Salt, sand and epiphytes were using tap water. Then, the samples were rinsed carefully with fresh water and freeze-dried. Dried alga sample was ground and sifted through a 50-mesh standard testing sieve. All chemicals and

* Corresponding author. Address: Department of Marine Life Science, Jeju National University, Jeju 690-756, Republic of Korea. Tel.: +82 64 754 3475; fax: +82 64 756 3493.
E-mail address: youjinj@jejunu.ac.kr (Y.-J. Jeon).

0887-2333/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2009.11.002

reagents used were of analytical and obtained from commercial sources.

The UV and FT-IR spectra were recorded on a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrometer and a SHIMAZU 8400s FT-IR spectrometer, respectively. NMR spectra were recorded on a Varian INOVA 400 MHz spectrometer. CD_3OD were used as a solvent for the NMR experiments, and the solvent signals were used as an internal reference. The HPLC was carried out on a YoungLin Instrument HPLC system equipped with a YoungLin acme 9000 UV/VIS detector and Autochrome software using C_{18} column (Waters Spherisorb® DOS-2 RP-18, 4.6 × 250 mm, 5 μm, Waters Co.).

2.2. Isolation of dieckol

The dried *E. cava* powder (500 g) was extracted three times with 80% methanol, and filtered. The filtrate evaporated at 40 °C to obtain the methanol extract, which was dissolved in water, then partitioned with ethyl acetate. The ethyl acetate fraction (45.65 g) was mixed with Celite. The mixed Celite was dried and packed into a glass column, and eluted in the order of hexane, methylene chloride, diethyl ether, and methanol. The diethyl ether fraction (26.69 g) was subjected to Sephadex LH-20 column chromatography using stepwise gradient chloroform/methanol (2/1 to 1/1 to 0/1) solvents system, and then finally purified by reversed-phase HPLC to give compound dieckol (275.8 mg). The purified compound was identified by comparing ^1H and ^{13}C NMR data to the literature report.

2.3. Cell culture

HUVECs were maintained in culture at 37 °C in an humidified atmosphere containing 5% CO_2, in an endothelial cell growth medium-2 EBM-2 supplemented with ascorbic acid, 2% fetal bovine serum (FBS), hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), human epidermal growth factor (hEGF), long R insulin-like growth factor-1 (R3-IGF-1), gentamicin sulfate (CA-1000) and heparin as described by the manufacturer (Clonetics, Walkersville, MD, USA).

2.4. Assay of neutral red cell viability

Cell viability was assessed by measuring the uptake of the supravital dye neutral red (Fautz et al., 1991). Cells (4×10^4 cells/well) in wells of 24-well plates were preincubated with glucose (5 mM or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of dieckol for 20 h. Thereafter, the medium was carefully removed from each well, and replaced with 0.5 ml of fresh medium containing 1.14 mM neutral red. After 3 h of incubation, the medium was removed, and then the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation in the presence of 1 ml of cell lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM dithiothreitol (DTT), and 1% (v/v) Triton X-100] containing 1% (v/v) acetic acid and 50% (v/v) ethanol at room temperature for 15 min. To measure dye uptake, the cell lysis products were centrifuged and the absorbance of the supernatant was measured spectrophotometrically at 540 nm.

2.5. Assay of lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) production Fraga et al., 1988. Cells (4×10^4 cells/well) in wells of 24-well plates were preincubated with glucose (5 mM or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of dieckol for 20 h. A 200 μl of each medium supernatant was mixed with 400 μl of TBARS solution then boiled at 95 °C for 20 min. The absorbance at 532 nm was measured and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve, TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

2.6. Assay of intracellular ROS levels

Intracellular ROS levels were measured by the dichlorofluorescein assay (Wang and Joseph, 1999). 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) can be deacetylated in cells, where it can react quantitatively with intracellular radicals (mainly hydrogen peroxide) to converted into its fluorescent product, DCF, which is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in oxidative stress. Cells (4×10^4 cells/well) in well of 24-well plates were preincubated with glucose (5 mM or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of dieckol for 20 h, after that the cells were washed with phosphate buffered saline (PBS) and incubated with 5 μM DCF-DA for 30 min at room temperature. Fluorescence was measured using a fluorescence plate reader.

2.7. Assay of nitric oxide (NO) levels

Cells (4×10^4 cells/well) in well of 24-well plates were preincubated with glucose (5 mM or 30 mM) for 48 h, and then incubated without or with the indicated concentrations of dieckol for 20 h. The nitrite accumulation in the supernatant was assessed by Griess reaction (Nath and Powledge, 1997). Each 50 μl of culture supernatant was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate absorbance reader, and a series of known concentrations of sodium nitrite was used as a standard.

2.8. Total and nuclear protein extracts

Cells were homogenized with ice-cold lysis buffer containing 250 mM NaCl, 25 mM Tris–HCl (pH 7.5), 1% (v/v) NP-40, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (10 μg/ml aprotinin, 1 μg/ml leupeptin). The cells were then centrifuged at 20,000*g* for 15 min at 4 °C. The supernatants were used as total protein extracts (Yamabe et al., 2007). For nuclear protein extracts, cells were homogenized with ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 15 mM $CaCl_2$, 1.5 M sucrose, 1 mM DTT, and protease inhibitor cocktail (10 μg/ml aprotinin, 1 μg/ml leupeptin). Then, the cells were centrifuged at 11,000*g* for 20 min at 4 °C. The supernatants were resuspended with extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 10 mM DTT, and protease inhibitor cocktail (10 μg/ml aprotinin, 1 μg/ml leupeptin). The samples were shaken gently for 30 min and centrifuged at 21,000*g* for 5 min at 4 °C. The pellets were used as nuclear protein extracts. The total and nuclear protein contents were determined by the Bio-Rad protein kit with BSA as the standard.

2.9. Immunoblotting

iNOS, COX-2 expressions and NF-κB p65 binding activity were determined by western blot analysis (Yamabe et al., 2007). Total protein (30 μg) for iNOS, COX-2 protein levels and nuclear protein (30 μg) for NF-κB were electrophoresed through 10% sodium dodecyl sulfate–polyacrylamide gel. Separated proteins were transferred electrophoretically to a pure nitrocellulose membrane,