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Chronic equol administration attenuates the antioxidant defense system and causes apoptosis in the mouse brain

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ABSTRACT

We investigated the effects of equol on the antioxidant defense system and apoptosis in the brains of mice administered equol at 5 or 25 mg/kg BW for 3 or 7 weeks. The effects of equol on the antioxidant defense system differed with the administration conditions. At 3 weeks, equol significantly inhibited lipid peroxidation and increased the catalase and total SOD activity in a dose-dependent manner, although equol did not have much effect on the GSH-related system. Following equol administration for 7 weeks, the level of TBARS was increased, while the catalase and total SOD activity were attenuated, although the difference was significant only at the higher dose. Moreover, at the higher dose, equol significantly down-regulated the GSH-related defense system. The GSH/GSSG ratio was decreased in a dose-dependent manner, as was the GSH-px and GR activity. As a result of these changes, apoptosis was induced in the mouse brain at both doses. The apoptosis process in the brain triggered by equol at the higher dose was consistent with a report that equol leads to apoptosis via p53 activation *in vitro*. Based on our results, chronic equol administration at a higher dose may disrupt the antioxidant defense system and induce apoptosis in the mouse brain.

1. Introduction

The brain is very sensitive to oxidative stress, because the brain has low-to-moderate activity with respect to enzymatic defense systems, compared with other organs, while the brain cells consume over 20% of the oxygen utilized by the body (Cooper, 1997; Ho et al., 1997a,b). Oxidative damage in brain occurs in most neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Bonilla, 2000; Moreira et al., 2005; Nunomura et al., 2007).

To against the oxidative stress, the human body has antioxidant defense systems, including both enzymatic and nonenzymatic mechanisms. The enzymatic defense system is composed of superoxide dismutase (Mn- and CuZn-SOD), catalase, and glutathione-related enzymes such as glutathione peroxidase (GSH-px), glutathione reductase (GR), and glutathione-S-transferase (GST). The consumption of natural antioxidants supports the endogenous antioxidant defense system. This has increased interest in flavonoids, which are widely distributed in most edible plants and composed of six major subclasses including flavones, flavonols, flavanones, flavanols, anthocyanidins, and isoflavones (Ross and Kasum, 2002). Many *in vitro* and *in vivo* studies have suggested that flavonoids have the potential to function as antioxidants (Kandaswami and Middleton, 1994; Bors et al., 1997). In addition, increasing evidence suggests that flavonoids at high concentrations may have pro-oxidant capacity (Cao et al., 1997; Yoshino et al., 1999; Galati et al., 2002).

Equol [7-hydroxy-3-(4′-hydroxyphenyl)-chroman] is a major metabolite of the isoflavone daidzein. Like isoflavones, equol has antioxidant activity and the ability to act as a hydrogen/electron donor; thus, equol can scavenge free radicals (Mitchell et al., 1998; Rüfer and Kulling, 2006; Yuan et al., 2007). Moreover, equol behaves as a more potent antioxidant than daidzein or genistein when measured *in vitro* (Hwang et al., 2003; Rüfer and Kulling, 2006). In particular, several studies have suggested that metabolites (such as equol) are responsible for the clinical effectiveness of isoflavones (Setchell et al., 2002; Cooke, 2006; Yuan et al., 2007).

Nevertheless, equol has gone relatively unnoticed compared with daidzein, and only a few *in vitro* and *in vivo* studies have assessed its activity. Therefore, we investigated the effects of chronic equol administration on oxidative stress and the antioxidant defense system in the mouse brain and whether it might induce apoptosis.

2. Methods and materials

2.1. Equol administration and sample preparation

For the present study, ICR female mice (Central Lab. Animal Inc., Seoul, Korea) were acclimated for 1 week under standard environmental condition with AIN 93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. After an

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adaptation period, mice were divided randomly into three treatment groups. Equol (LC Laboratories®, MA, USA) was suspended in water and administered by oral gavages to two groups at 5 and 25 mg/kg body weight (BW) for 7 weeks. Mice in the remaining group were given the vehicle alone (water) as orally administration. Animal care in this study conformed to *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. At the end of experiment, mice were rapidly anesthetized using ether and then after their brains were isolated, blotted, weighed, frozen in liquid nitrogen and stored at −70 °C until assayed. For assay, each brains were homogenized for about 45 s in 9 volume of ice-cold 10 mM phosphate buffered saline (PBS, pH 7.4) containing 1.15% KCl. Homogenates were centrifuged at 800*g* to remove cell debris and nuclei; the supernatants were centrifuged at 10,000*g* for 10 min and transferred the portions of the post-mitochondrial fraction. To obtain the cytosolic fractions for measurement SOD activity, the remaining supernatant was centrifuged further at 105,000*g* for 45 min using a 50 Ti rotor in a Beckman model L90 ultracentrifuge. Post-mitochondrial and cytosolic fractions were stored at −80 °C in aliquots until analysis within one week.

2.2. Determination of lipid peroxidation

TBARS value was determined by measuring the concentration of malondialdehyde according to the method of Ohkawa et al. (1979) and its calculated according to the molar absorption coefficient of MDA, $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 535 nm. Protein concentration was determined by Bradford protein assay kit II (Bio-rad, Laboratories, CA).

2.3. Determination of catalase and total SOD activity

Catalase activity was assayed by the method of Aebi (1984). Catalase activity was calculated as nmol of H_2O_2 decomposed/min/mg/protein. SOD activity was assayed according to the pyrogallol autoxidation method of Marklund and Marklund (1974). Each unit of SOD activity was defined as the quantity of enzyme that inhibited autooxidation of pyrogallol by 50% under experimental conditions.

2.4. Determination of GSH-related system

The GSH content was measured with use of *o*-phthalaldehyde as a fluorescent reagent according to method of Hissin and Hilf (1976). For GSSG measurement, each sample was incubated with *N*-ethylmaleimide to interact with GSH present in the tissue homogenate. The GSH and GSSG content (μg/mg protein) were obtained from a standard curve and then after the GSH/GSSG ratio was calculated. Activities of glutathione peroxidase and reductase were determined with a spectrophotometer by measuring the disappearance of NADPH at 340 nm, based on the methods of Flohé and Günzler (1984) and Carlberg and Mannervik (1985), respectively. The enzyme activities of glutathione peroxidase and reductase were defined as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^{-3}\ M^{-1}\ cm^{-1}$.

2.5. Immunoblotting assay

SDS–PAGE Immunoblotting was performed as previously described in Choi and Lee (2004). Antibodies against, Bcl-2, Bcl-xL, Bax, and β-actin were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., CA). The specific protein bands were detected by Opti-4CN Substrate kit (Bio-rad).

2.6. Culture of primary cortical neurons and hepatocytes

Primary cortical neuronal and hepatocytes cultures were prepared from the dissected cortical hemispheres of 16–17 day old Sprague-Dawley rat embryos and male Sprague-Dawley rats, respectively. Sprague-Dawley rats (230–260 g; Daehan Biolink, Chungbuk, Korea) were individually housed in cages kept at 22 ± 2 °C, with 40–50% relative humidity and controlled lighting under a 12-h light:dark cycle. Rats were fed an AIN 93 M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. Hepatocytes were prepared using a two-step collagenase perfusion method (Seglen, 1976) and were cultured in Ham's F-12/DMEM (Invitrogen, Carlsbad, CA, USA) medium supplemented with 100 U/ml penicillin and 70 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cortical neurons were prepared according to the method of previously report (Choi and Lee, 2004) and cultured with MEM solution supplemented with 2 mM L-glutamate, 10% (v/v) fetal calf serum, and 10% horse serum (Gibco-BRL Life Tech., Rockville, MD, USA) at same condition as above. Cell viability was confirmed by Trypan blue exclusion. Hepatocytes and cortical neurons were plated at a density of $1.0–1.5 \times 10^6$ cells/well on 6-well plates. To analyze the caspase-3 activity, cells were treated with either daidzein or vehicle alone at various concentration (ranging from 1 to 100 μM) for 24 h. Equol was dissolved in DMSO (final concentration 0.1% in medium).

2.7. Determination of caspase-3 activity

Caspase-3 activity was measured by EnzChek Caspase-3 Assay Kit (Invitrogen Corporation, CA, USA). The basis for the assay is the aminomethylcoumarin (AMC)-derived substrate Z-DEVD-AMC.

2.8. Statistical analyses

Data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test (Sigma Stat., Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Lipid peroxidation inhibition by equol administration

In mice administered equol for 3 weeks, the level of thiobarbituric acid-reactive substance (TBARS) was decreased by 7.22% and 15.33% at a dose of 5 and 25 mg/kg body weight (BW), respectively, although the difference was significant at the higher dose only (Fig. 1). Conversely, although no change was observed in mice administered a lower dose of equol for 7 weeks, the TBARS level was significantly higher, by around 12%, at the higher dose compared to the control.

3.2. Down-regulation of antioxidant defense system by equol administration

The catalase and total superoxide dismutase (SOD) activity increased significantly after 3 weeks of equol administration compared to the control group in a dose-dependent manner (Fig. 2A). Equol did not significantly affect the glutathione/oxidized glutathione (GSH/GSSG) ratio or glutathione peroxidase (GSH-px) or glutathione reductase (GR) activity, although a tendency toward a slight increase in the dose–response pattern was observed for the GSH/GSSG ratio (Fig. 2B).

In mice administered equol for 7 weeks, at the higher dose, equol significantly reduced the catalase and total SOD activity by 26.75% and 17.67%, respectively, compared to control levels (Fig. 3A). In addition, a dose-dependent reduction in the GSH/GSSG ratio was observed (Fig. 3B). Compared to the control value, a decrease of 19.80% was detected at the higher dose. Equol also significantly decreased the GSH-px and GR activity by 15.18% and 25.54%, respectively, compared to the control group.

3.3. Apoptosis induced by equol administration

In the brains of mice administered equol for 7 weeks, activation of p53 was observed and was more pronounced at the higher dose

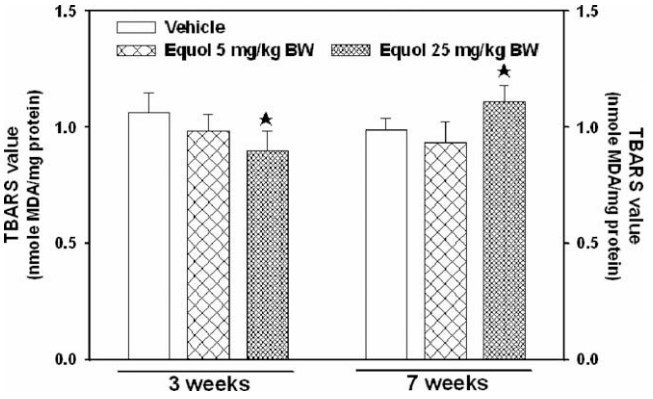

Fig. 1. Lipid peroxidation. Equol was administered orally at two levels, 5 and 25 mg/kg BW/day for 3 and 7 weeks. Values are mean ± S.D. *$P < 0.05$, significantly difference between control (vehicle-only) and equol-administered groups.