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Daidzein Has Anti-Oxidant Activity in Normal Human Kidney Tubular HK-2 Cells via FOXO3/SOD2 Pathway

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Abstract The anti-oxidant activity of daidzein was accessed by checking the expression level of superoxide dismutase 2 (SOD2) in HK-2 cell, a kidney proximal tubular cell line. SOD2 expression was increased in HK-2 cells treated by daidzein with a dose and time-dependent manner. Daidzein reduced the reactive oxygen species (ROS) level (about 60% compared to DMSO control with $p = 0.005$) in HK-2 cells. Daidzein caused FOXO3 to be translocated from cytosol into nucleus. Down-regulation of FOXO3 by transfection of siRNA against FOXO3 attenuated the expression of SOD2 and ROS level ($p = 0.006$) by daidzein. Taken together, this study has shown that daidzein displays anti-oxidant activity in HK-2 kidney proximal tubular cell line through up-regulation of FOXO3/SOD2 signaling pathway.

Keywords: *daidzein, anti-oxidant, HK-2, FOXO3, SOD2*

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1. Introduction

Oxidative damage in cells is known to result from the increased level of free radicals and reactive oxygen species and the decreased level of anti-oxidant related proteins and molecules. The etiological and pathological role of the oxidative stress in cells has been elucidated in various diseases [1]. Dongyan *et al.* showed the anti-oxidant activity of the water extract of mung bean sprouts by measuring in vitro anti-oxidant assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and human skin patch test [2]. These recent studies imply the extracts of mung bean sprouts are safe and can be used as additives in anti-aging cosmetic products. Also, there are two previous studies demonstrating the anti-oxidant effects of soy extract in rats and mice model. Teixeira *et al.* performed analysis of reactive species quantification in rats treated with soy extract and suggested that the soy extract-treated group showed lower reactive species level compared to control group [3]. Georgetii *et al.* evaluated the anti-oxidant activity of soybean extract by measuring ROS level such as 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced hydrogen peroxide (H(2)O(2)) and malondialdehyde (MDA) in mice treated topically with soybean extract formulation [4]. They concluded that the formulations containing soybean extract has anti-oxidant activity and may be used as one of a topical source for reducing oxidative stress in the skin. In addition, Aras *et al.* proved that superoxide dismutase expression levels in the brain tissue of rats with ischemia was reduced but increased after daidzein administration by biochemical and immunohistochemical tests [5]. They suggested that daidzein may have neuroprotective effects on ischemic brain by activating superoxide dismutase and decreasing ROS generation.

Daidzein is one of isoflavones that are present with large amount in soybeans and well-known bioactive compounds to be involved in human health state [6]. Diet of soybean products have been reported to prevent incidence or severity of chronic human disorders such as breast, prostate cancer, and osteoporosis. Thus, diet of soybean related foods might provide us with the good health condition **[7]**. The prevention of oxidative stress by taking food containing lot of anti-oxidants can help human to maintain good health and prevent human from diseases caused by oxidative stress [8]. So far, a number of studies about the anti-xanthine oxidase and anti-oxidant activity of flavonoids including daidzein have been extensively reported. Generally, flavonoids are known to have benefits to human health by showing a number of biological functions including antagonizing and inhibiting of ROS [9].

Superoxide dismutase 2 (SOD2) is one of the most important defense enzymes against oxidative stress in cells such as oxygen radicals. In addition, SOD2 can convert superoxide into peroxide for scavenging them, which is finally removed by catalase [10]. Thus, a deficient expression or low enzyme activity of SOD2 may result in a condition where anti-oxidant defense mechanism in cell is not sufficient to balance the levels of oxidative stress. It will be good standard consideration whether a specific

compound can induce SOD2 expression level to be up-regulated in human cell lines to protect against oxidative stress.

In this study, for the demonstration of the anti-oxidant mechanism of daidzein that is rich in bean sprout, we evaluated the effects of daidzein on the anti-oxidant activity by measuring SOD2 expression level in HK-2 cells, a human kidney proximal tubular cell lines.

2. Materials and Methods

2.1. Cell Lines

HK-2 cells (from ATCC) were grown in Keratinocyte Serum Free media supplemented with 10% fetal bovine serum, bovine pituitary extract (BPE, 0.05 mg/ml) and human recombinant epidermal growth factor (hEGF, 5 ng/ml), and 1% Streptomycin/Penicillin at 37°C in a humidified incubator containing 5% CO_2 in air.

2.2. WST-1 Cell Viability Assay

A 200 µl media with cells (1 X 10^3) was seeded into each well in 96-well plate and grown for 18 h at 37°C in a humidified incubator containing 5% CO_2. Then, the indicated dose of daidzein was added into each well and incubated for 72 h. 20 µl assay substrate was added into each well and incubated for 4 h. The optical density at 460 nm was measured by a microplate reader.

2.3. Cell Counting Assay

Cells (1 X 10^4) were seeded in 6-cm dishes and incubated at 37°C in a humidified incubator containing 5% CO2 in air incubator for 18 h. After incubation, cells were treated with DMSO as control vehicle and the indicated concentration of daidzein (50 µM) for 0, 24, 48 and 72 h. Each day, cell numbers were measured by using a hemocytometer.

2.4. Cytoplasmic and Nuclear Protein Fractionation

Cells from each condition were trypsinized, centrifuged, washed, re-suspended in a cytoplasmic fractional buffer and incubated at 4°C for 30 min on a rotator. The cell suspension was centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was collected for cytoplasmic fraction. The nuclear pellet was washed twice with the washing buffer. The remaining pellet was re-suspended with a nuclear fractional buffer and incubated at 4°C for 30 min on a rotator. The nuclear suspension was centrifuged at 13,000 rpm for 30 min at 4°C, the supernatant was collected for nuclear fraction.

2.5. Western Blotting Analysis

Cells were washed (PBS, 2 times) and lysed with RIPA buffer with phosphatase and protease inhibitors. Cell lysates were centrifuged (10,000 X g, 4°C, 10 min). Proteins were resolved on 10% SDS–PAGE gels and blotted onto nitrocellulose membranes. Membranes were blocked in 3% bovine serum albumin for 1 h at room temperature, and probed with antibodies. Then membranes were probed with horse radish peroxidase (HRP)-tagged anti-mouse IgG antibodies at room temperature for 1 h. Chemiluminescence was detected using the ECL substrate.

2.6. siRNA Transfection

Control siRNA and siRNAs against FOXO3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with each siRNA using Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instruction.

2.7. Measurement of ROS

A 200 µl media with cells (1 X 10^5) was seeded into each well in 6-well plate and grown for 18 h at 37°C in a humidified incubator containing 5% CO2. Then, cells were treated with daidzein (100 µM) for 30 min at 37°C, and then loaded with Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, MA) in a 5% CO2, 37°C incubator for 1 h. The fluorescent intensities were measured with a flow cytometer using FL2 channel.

2.8. Immunofluorescence Analysis

For the immunofluorescence analysis, HK-2 cells were treated with daidzein (50 µM) or vehicle (DMSO) for 24 h, fixed, and permeabilized. After blocking with BSA, cells were incubated with a primary antibody against FOXO3 followed by Alexa 488-conjugated anti-mouse secondary antibody. After counterstaining with DAPI, fluorescence images were captured with a confocal microscope.

3. Results and Discussion

To test the potential anti-oxidant activity of daidzein in HK-2 cells, we measured ROS level in HK-2 cells treated with daidzein. As shown in Figure 1. A, daidzein treatment reduced ROS level to about 60% of compared to DMSO vehicle control. Also, we performed western blotting of SOD2 expression in HK-2 cells treated with daidzein. As shown in Figure 1. B and C, daidzein caused the expression of SOD2 in HK-2 cells with dose and time-dependent manner. However, as shown in the Figure 1 B and C, neither SOD1 nor SOD3 was not significantly changed after treatment of daidzein. Thus, daidzein might have the possible anti-oxidant activity in HK-2 cells through increasing only SOD2 expression. Furthermore, the effective dose of daidzein in HK-2 cells did not alter the cell growth up to 72 h incubation (Figure 1. D and E).

For elucidating molecular working mechanism of daidzein to regulating SOD2 expression level, we checked FOXO3 expression pattern in HK-2 cells treated with daidzein since FOXO3 is well-known transcription factor of SOD2. As shown in Figure 2. A, daidzein treatment induced the translocation of FOXO3 from cytosol into nucleus. At the same time, expression of FOXO3 in cytosol was decreased but expression of FOXO3 in nucleus was increased, which suggested that FOXO3 could have the transcriptional ability to regulate SOD2

expression. We confirmed FOXO3 translocation by immunofluorescence analysis of FOXO3 in HK-2 cells treated with daidzein (Figure 2. B). Also, we tested that FOXO3 is required for being responsible for SOD2 expression caused by daidzein treatment in HK-2 cells by transfecting HK-2 cells with control or FOXO3 siRNA. As shown in Figure 2. C, after transfecting HK-2 cells with siRNA against FOXO3, SOD2 expression was not increased so much as in HK-2 cells transfected with control siRNA. Furthermore, we measured ROS level in HK-2 cells transfected with siRNA against FOXO3 and treated with daidzein or DMSO by ROS detection kit. As shown in the Figure 2. D, compared to control siRNA transfection, ROS level in HK-2 cells transfected with siRNA against FOXO3 was not significantly changed after treatment of daidzein suggesting that daidzein exert anti-oxidant effects via FOXO3/SOD2 pathway. Thus, SOD2 expression by daidzein treatment in HK-2 cells could be mediated transcriptionally by FOXO3.

HK-2 cells is normal kidney cell line from proximal tubular of kidney. This cell line can be one of the best model systems to investigate a working mechanism of a specific chemical in cells. Although there are lots of cell lines that might be tried to check for this purpose, they are usually originated from cancer tissue, so we are not quite sure how the signaling pathway is altered than normal tissue situation [11]. Recently, Agharazii *et al*. reported that the expression of antioxidant enzymes such as SOD1, SOD2, Gpx1, and Prdx1 was reduced in rat model for chronic kidney disease (CKD) accompanied with production of ROS. Thus, ROS generation are associated with CKD via lower expression of anti-oxidant enzymes including SOD2. Daidzein could induce SOD2 expression but has no cytotoxicity against HK-2 cell lines.

To the best of our knowledge, this is the first report for the evaluation of the anti-oxidant mechanism of daidzein. For elucidating the detail molecular working mechanism how daidzein causes the up-regulation of SOD2 expression, we might need to focus on FOXO3 expression in HK-2 cells by daidzein treatment since FOXO3 is transcriptional factor responsible for regulation of SOD2 that was increased by daidzein treatment in HK-2 cells (Figure 2. A and B) [12]. In summary, we have validated that daidzein has the anti-oxidant activity which could suggest the potential use daidzein as one of foodstuff preventing the oxidative-related disease in human.

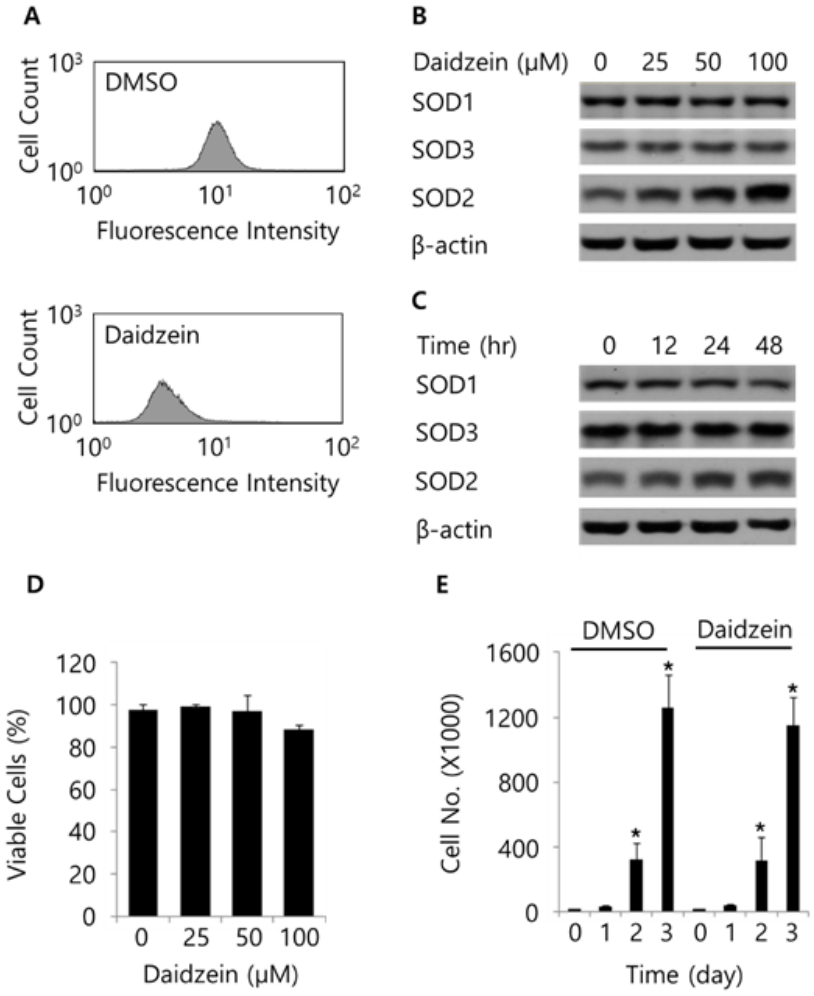

Figure 1. Daidzein has anti-oxidant activity through up-regulation of SOD2 expression in HK-2 cells. A) Decreased ROS level in HK-2 cells treated with daidzein. ROS level in HK-2 cells was measured by ROS detection kit B) Treatment of daidzein in HK-2 cells caused up-regulation of SOD2 expression with dose-dependency. Western blotting analysis of SOD1, SOD2, and SOD3 expression in HK-2 cells. C) Treatment of daidzein in HK-2 cells caused up-regulation of SOD2 expression with time-dependency. Western blotting analysis of SOD1, SOD2, and SOD3 expression in HK-2 cells. β-actin was used for loading control. D) Dose-dependent WST-1 assay showed that daidzein has no cytotoxicity on HK-2 cells. E) Cell counting assay was performed to check daidzein treatment in HK-2 cells affect the cell growth compared to DMSO control for 0, 24, 48, and 72 h. Significant between time group are indicated (* means $P < 0.05$). , error bars represent standard deviation, and the statistical test paired t-test

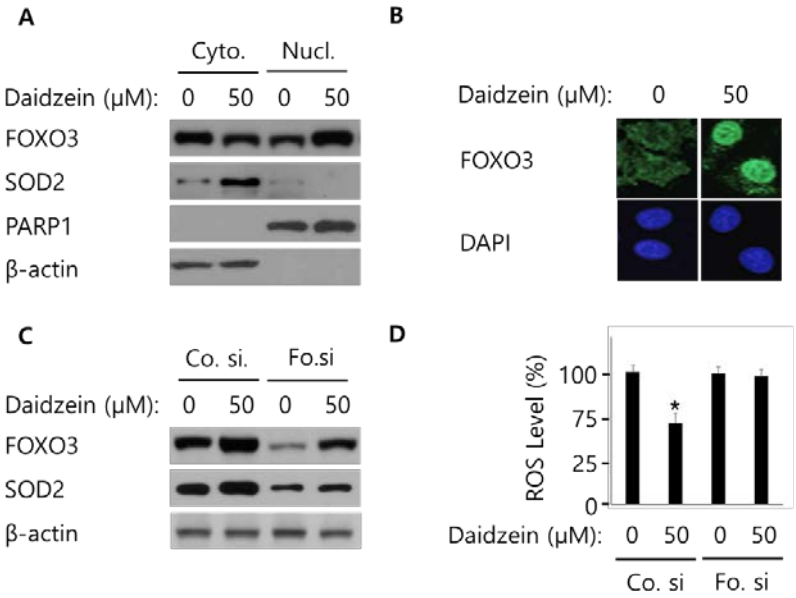

Figure 2. Involvement of FOXO3 to mediate SOD2 expression by daidzein. A) Protein from HK-2 cell treated with daidzein was prepared by the cytosol and nucleous fractional method described in material and method section. Then, FOXO3 and SOD2 expression level was analyzed by Western blotting. β-actin was used for cytosol loading control and PARP1 was for nucleous loading control. B) Immunofluorescence analysis of FOXO3 in HK-2 cells treated with daidzein was performed by the method described in the methods. C) siRNA against FOXO3 was transfected into HK-2 cells followed by daidzein treatment. Then, FOXO3 and SOD2 expression level was analyzed by Western blotting. β-actin was used for loading control D) siRNA against FOXO3 was transfected into HK-2 cells followed by daidzein treatment. Then, ROS level in HK-2 cells was measured by ROS detection kit

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Conflict of Interests

The author declares that there is no conflict of interests.

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