Original Article

Effects of Equol on Oxidized Low-Density Lipoprotein-Induced Apoptosis in Endothelial Cells

Masumi Kamiyama¹, Yoshimi Kishimoto², Mariko Tani², Kazunori Utsunomiya³, and Kazuo Kondo²

¹Food Processing Technology Division, Department of Food Technology, Nagano Prefecture General Industrial Technology Center, Nagano, Japan

²Institute of Environmental Science for Human Life, Ochanomizu University, Tokyo, Japan

³Division of Diabetes, Metabolism and Endocrinology, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan

Aim: Equol is the main active product of daidzein metabolism, produced via specific microflora in the gut. This study aimed to clarify the effects of equol on oxidized low-density lipoprotein (OX-LDL)-stimulated apoptosis in human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were cultured in the presence of OX-LDL, and cell apoptosis was monitored by evaluating of DNA fragmentation and the production of cytoplasmic histone-associated DNA fragments. We simultaneously evaluated the level of cellular superoxide and nitric oxide (NO) and the effects of the anti-oxidant activity of equol on apoptosis.

Results: We found that equol inhibited the induction of apoptosis in response to exposure of HUVECs to OX-LDL. Treatment of cells with equol led to a significant reduction in superoxide production by NAD(P)H oxidase and also to a significant increase in NO production. We further observed an effect of equol on the suppression of OX-LDL uptake.

Conclusions: These results suggested that equol might contribute to a reduced level of OX-LDL-stimulated apoptosis linked to the reduced generation of intracellular reactive oxygen species (ROS).

J Atheroscler Thromb, 2009; 16:239-249.

Key words; Equol, Endothelial dysfunction, Oxidized low-density lipoprotein, Apoptosis

Introduction

Modifications of low-density lipoprotein (LDL), such as oxidation, are thought to be an important mediator of vascular endothelial dysfunction and atherosclerosis. LDL oxidation can be mediated by various processes, such as the action of transition metal ions (copper or iron), nitric oxide/superoxide radicals, or the action of peroxidase enzymes¹⁻². It has previously been demonstrated that scavenger receptors for OX-LDL in human umbilical vein endothelial cells

(HUVECs) facilitate the uptake of OX-LDL and mediate several of its biological effects³⁻⁵. Specifically, the binding of OX-LDL to LOX-1 results in a significant increase in the generation of reactive oxygen species (ROS) by NAD(P)H oxidase. Furthermore, increased expression and activity of receptors in response to OX-LDL exposure is associated with decreased eNOS expression and endothelial dysfunction⁶. Shin *et al.* reported that remnant lipoprotein particles (RLPs) stimulated NAD(P)H oxidase-dependent superoxide formation and the induction of cytokines in HUVECs via LOX-1 activation, leading to reduced cell viability and DNA fragmentation⁷. Intracellular free radical production has been suggested to contribute to OX-LDL-induced endothelial cell dysfunction.

Several polyphenol compounds have recently attracted interest as anti-oxidant agents. Isoflavone is a flavonoid, a family of polyphenol compounds found

Address for correspondence: Masumi Kamiyama, Nagano Prefecture General Industrial Technology Center, Food Processing Technology Division, Department of Food Technology, 205-1 Nishibanba Kurita, Nagano City, Nagano 380-0921, Japan

E-mail: kamiyama-masumi-r@pref.nagano.jp

Received: August 28, 2008

Accepted for publication: December 8, 2008

Fig. 1. Chemical structures of soy isoflavones and metabolite of daidzein.

in common food items derived from plants (**Fig. 1**). Isoflavones are also an important class of phytoestrogens, which are found at extremely high levels in soy. Recently, the isoflavones daidzein and genistein have been shown to have beneficial physiological effects on cardiovascular disease, blood pressure and angiogenesis⁸⁻¹². More recently, however, attention has shifted to isoflavone metabolites. Equol is the main active product of daidzein metabolism, and is produced by specific microflora in the gut (**Fig. 1**). Equol has a longer half-life and greater biological activity, including superior antioxidant activity, than daidzein; however, whilst most animals produce equol following soy consumption, as high as 50% of the adult human population does not¹³. This ability by a percentage of the human population to produce equol may explain the positive health benefits of soy consumption observed in clinical studies¹⁴; however, the role of equol and the mechanism of its effect on endothelial dysfunction remain to be clarified.

In the present study, we report the effects of equol on the response of endothelial cells to OX-LDL exposure *in vitro*. We also demonstrate the effect of equol upon the induction of endothelial cell apoptosis.

Methods

Isolation of LDL

LDL was isolated by ultracentrifugation as reported previously¹⁵. Blood samples were collected in

EDTA-containing tubes from healthy normolipidemic adult women after an overnight fast. Plasma was prepared by centrifugation at 3,000 rpm (2,000 *xg*) for 15 min at 4°C. LDL was isolated from 2.2 mL plasma by single-spin density gradient ultracentrifugation (100,000 rpm (41,700 *xg*), 40 min, 4°C) with a TLA-100.4 fixed-angle rotor (Beckman, Palo Alto, CA). The density of the plasma was adjusted to 1.21 g/mL by adding 0.715 g KBr to 5.1 mL ultracentrifuge tubes and then filling the air space of the tubes with about 2.9 mL of *d*=1.006 g/mL saline-EDTA buffer. The protein content of LDL was determined by a commercial test kit (Bio-Rad, California, USA) using bovine albumin as the standard. Before the start of the oxidation experiments, LDL samples were diluted with phosphate-buffered saline (PBS) to give a final concentration of 100 µg/mL LDL protein.

Preparation of OX-LDL

Native LDL (100 µg/mL) was oxidized by incubating in the presence of copper sulfate (5 µM) at 37°C. Oxidation was stopped by the addition of EDTA (100 µM). The final preparation was dialyzed against 150 mM NaCl containing 0.1 mM EDTA and filter-sterilized. The degree of LDL oxidation was confirmed by analysis of the formation of thiobarbituric acid reactive substance (TBARS assay kit; ZeptoMetrix, NY, USA). Conjugated diene formation during LDL oxidation was continuously monitored by measuring changes in absorbance at 234 nm using a spectrophotometer (DU-7400; Beckman, Palo Alto, CA). The

change in the surface charge of LDL protein was evaluated by the increase in its electrophoretic mobility, determined using the TITAN GEL Lipoprotein Kit (Helena Laboratories, Saitama, Japan).

Cell Culture

HUVECs (CELL APPLICATIONS, San Diego, CA) were cultured in ENDOTHELIAL CELL GROWTH MEDIUM (211-500; CELL APPLICATIONS). Cells were grown to confluence at 37°C in 5% CO₂ and used for experiments at no greater than passage 6.

DNA Fragmentation Assay

Cells were exposed to OX-LDL for 48 h, with or without preincubation with equol (for 6 h). We further examined the effect of *N*-acetyl cysteine (NAC; Wako, Osaka, Japan), L-NAME (*N*^G-Nitro-L-arginine methyl ester; Dojindo, Kumamoto, Japan) or estrogen receptor inhibitor (ICI 182,780; Tocris, Ballwin, MO, USA) on OX-LDL stimulation.

The culture medium was removed and centrifuged at 2,000 rpm for 5 min to collect any detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) and then pooled with the pellet composed of detached cells. After incubation at 4°C for 15 min, lysates were incubated with 10 µg of 10 mg/mL RNase A for 1 h at 37°C followed by 10 µL of 20 mg/mL proteinase K for 2 h at 50°C. DNA was extracted using chloroform: phenol: isoamyl alcohol (25:24:1). It was then precipitated overnight with 1 volume of isopropyl alcohol at -20°C, electrophoresed through a 2% agarose gel, and then visualized under UV light after staining with ethidium bromide.

Furthermore, apoptotic cell death was analyzed by a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) (Cell Death Detection ELISA^{PLUS}; Roche, Mannheim, Germany).

Real-Time Quantitative RT-PCR

RNAs were extracted from cells using the Nucl-eospin purification system (Macherey-nagel, Duren, Germany) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Expression of the genes encoding human NAD(P)H oxidase subunits (p22phox, p47phox, p67phox) and eNOS in HUVECs were examined by real-time quantitative RT-PCR (Applied Biosystems 7,300 real-time PCR system;

Table 1. Primers for real-time quantitative RT-PCR

Gene name (human)	Forward (5' to 3')	Reverse (5' to 3')
p22phox	gtacttttggtgcctactcca	cggcccgaacatagtaattc
p47phox	ttgagaagcgcttcgtaccc	cgtcaaacacttgggagct
p67phox	cagttcaagctgtttgcctg	ttctggccagctgagccac
eNOS	cactcccatgactttggtgttcggc	cttcgtgtagcgttgctgatcccg
36B4	aagaacacatgatgcgcaag	ccttattggccagcaacatgctc

Applied Biosystems, Tokyo, Japan) with SYBR Green detection. Results were normalized against those from the human 36B4 gene. Primers for the genes studied are indicated in **Table 1**. Each PCR contained 2x Power SYBR Breen Master Mix (Applied Biosystems, CA, USA), 800nM gene-specific primer and 50 ng template DNA in a 20 µL reaction volume. The thermal profile was 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1min. Dissociation profiles of the amplified products were run to evaluate nonspecific amplification.

Western Blot Analysis

HUVECs were lysed in 250 mM sucrose, 10 mM EGTA, 1 mM EDTA, 20 mM Tris-HCl (pH7.5), 1 mM PMSE, 0.1% leupeptin, 0.1% aprotinin. The lysed cells were centrifuged at 100,000 xg to remove the cytosolic fraction. Equal amounts of proteins were separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA), and the membrane was blocked in 5% non-fat dry milk in Tris buffer (pH 8.0) containing 0.05% Tween-20 (v/v), and then probed with an anti Rac1 antibody (diluted 1:1000; Chemicon, Temecula, CA, USA) overnight. After washing, the membrane was incubated with an anti-mouse-horseradish peroxidase-conjugated antibody (diluted 1:2000; Amersham Pharmacia, Little Chalfont, UK) for 1 h. Immunoreactive bands were detected with a chemiluminescence detection system (ECL Plus Western Blotting Detection System; Amersham Pharmacia).

Fluorescence Detection of Intracellular Superoxide and NO

For the assay of intracellular superoxide production, HUVECs were incubated with 10 µM dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA) in the presence of L-NAME for 15 min. A fluorescence reader (spectrofluorophotometer RF-1500; Shimadzu, Kyoto, Japan) with 518 nm excitation and 605 nm emission filters was used to detect of DHE.

For the assay of intracellular NO production,

Fig. 2. Determination of LDL.

LDL was incubated with 5 μ M CuSO₄ in PBS for various periods of time at 37°C. The degree of oxidation was quantified by three different methods: A: Electrophoretic mobility through an agarose gel; B: TBARS; C: Conjugated diene formation. Values represent the mean $\pm$ S.D. of data from three independent experiments. * p < 0.01.

HUVECs were incubated with 10 μ M 4, 5-diamino-fluorescein diacetate (DAF-2DA; Molecular Probes) for 15 min. A fluorescence reader with 495 nm excitation and 515 nm emission filters was used to detection of DAF-2DA.

Background fluorescence from cell-free incubation was subtracted from the data. Data are expressed as fluorescence intensity.

Endothelial Uptake of OX-LDL

OX-LDL was labeled basically as previously described¹⁶. OX-LDL was incubated at 37°C with Dil (Molecular Probes) in lipoprotein-deficient serum (Sigma, St Louis, MO, USA) for 15 h. Labeled OX-LDL was purified by ultracentrifugation over a KBr gradient and dialyzed using an Amicon® Ultra-15 Centrifugal Filter Device (Millipore, Billerica, MA, USA). Dil-labeled oxidized LDL was used to visualize the uptake of OX-LDL by endothelial cells. After incubation of cells with equol or a monoclonal antibody against LOX-1¹⁷ (monoclonal antibody 23C11 can be used to neutralize of the biological activity of LOX-1; HyCult Biotechnology, Uden, The Nether-

lands), cells were treated with 0.03 mg protein/mL Dil-labeled OX-LDL for 4 h or 0.03 mg protein/mL unlabeled OX-LDL to determine specific uptake of OX-LDL. After incubation, the cells were rinsed with PBS containing 0.05% Tween20 and fixed in 4% ice-cold formaldehyde for 30 min. The cellular distribution of Dil was analyzed by confocal laser scanning microscopy (FluoView FV300 Confocal Microscope; Olympus, Tokyo, Japan).

Statistical Analysis

Values represent the mean $\pm$ S.D. Data for cell viability, real-time quantitative RT-PCR and intracellular ROS were analyzed by one-way analysis of variance, followed by Scheffe's test to evaluate statistical differences.

Results

Determination of OX-LDL and Induction of Cell Apoptosis in HUVECs

We first oxidized native LDL by incubating in the presence of copper sulfate. We next evaluated the

Fig. 3. OX-LDL-induced cell apoptosis of HUVECs.

OX-LDL showed oxidation time and concentration-dependent cytotoxicity in HUVECs. A: With oxidation time of over 15 h, the death of HUVECs at 48 h of OX-LDL (50 µg/mL) incubation was significantly increased. Values represent the mean $\pm$ S.D. of data from five independent experiments. B: OX-LDL (oxidation time: 15 h) induced apoptosis in a concentration-dependent manner. Values represent the mean $\pm$ S.D. of data from three independent experiments. * $p < 0.01$.

degree of LDL oxidation by evaluating its electrophoretic mobility (**Fig. 2A**), and by the TBARS assay (**Fig. 2B**), and the conjugated diene assay (**Fig. 2C**). Compared to native LDL, OX-LDL exhibited a greater mobility through an agarose gel (Titan Gel), and this greater mobility correlated with the degree of oxidation. In addition, the oxidation of LDL increased the production of TBARS and conjugated diene.

As shown in **Fig. 3**, OX-LDL was cytotoxic to HUVECs in a manner dependent upon both oxidation time and concentration. Treatment of HUVECs with OX-LDL exposed to an oxidation time of greater than 10 h led to a significant increase in cell death at 48 h post-treatment (**Fig. 3A**). Furthermore, OX-LDL (oxidation time: 15 h) induced apoptosis of HUVECs in a concentration-dependent manner (**Fig. 3B**).

For the remaining experiments, we used OX-LDL that had been oxidized for 15 h at a final concentration of 50 µg/mL.

Effect of Equol on OX-LDL-Stimulated Apoptosis

Treatment of HUVECs with equol alone had no effect on the induction of apoptosis but such treatment inhibited the induction of apoptosis in response to treatment of cells with OX-LDL. Treatment of HUVECs with either the antioxidant, *N*-acetyl cyste-

ine (NAC), or equol suppressed the induction of apoptosis by OX-LDL. Co-treatment of cells with *N*^G-Nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS) had no effect upon OX-LDL-stimulated apoptosis. These results suggest that inhibition of NO production antagonized the effect of equol on the suppression of OX-LDL-stimulated apoptosis (**Fig. 4A, B**).

OX-LDL Induces the Production of Superoxide by NAD(P)H Oxidase

Because NAC suppressed the induction of cell apoptosis by OX-LDL, we next examined the effects of OX-LDL treatment on NAD(P)H oxidase-dependent superoxide production and eNOS-dependent NO production.

Real-time quantitative RT-PCR analysis revealed that treatment of HUVECs with OX-LDL led to enhanced expression of p22phox and p47phox subunits, but not the p67phox subunit, of NAD(P)H oxidase. The increased expression of these genes was strongly suppressed by co-treatment with either equol in a dose-dependent manner. Treatment of cells with equol alone had no effect upon the expression of these genes (**Fig. 5A**).

We next evaluated the localization of the

Fig. 4. Effect of equol on OX-LDL-induced cell apoptosis in HUVECs and the effect of free radical scavengers.

HUVECs were preincubated with equol, NAC, or L-NAME as indicated prior to exposure of cells to OX-LDL for 48 h. A: Cells were harvested and DNA was extracted for fragmentation analysis. B: measurement of apoptotic cell death. Values represent the mean $\pm$ S.D. of data from three independent experiments. * $p < 0.01$.

Fig. 5. OX-LDL-induced NAD(P)H oxidase-dependent superoxide production.

A: NAD(P)H oxidase subunit (p22phox, p47phox, and p67phox) mRNA was examined by real-time quantitative RT-PCR. Values represent the mean $\pm$ S.D. of data from three independent experiments. * $p < 0.01$. B: NAD(P)H oxidase subunits, Rac1 translocation from cytosol to membrane, Western blot of Rac1 protein in the membrane fraction was performed. C: OX-LDL-induced intracellular superoxide production. Values represent the mean $\pm$ S.D. of data from three independent experiments. * $p < 0.05$.

NAD(P)H oxidase subunit, Rac1, in the membrane fraction by Western blot analysis, to monitor the translocation of these subunits from the cytosol to the membrane in response to exposure of cells to OX-LDL (**Fig. 5B**). We confirmed that OX-LDL treatment alone increased Rac1 protein translocation, whereas pretreatment of HUVECs with equol suppressed the translocation of these subunits in response to OX-LDL exposure.

We next examined the effects of OX-LDL on intracellular superoxide production by evaluating DHE fluorescence (**Fig. 5C**). We observed that superoxide production by HUVECs was elevated when the cells were incubated for 24 h in medium containing 50 $\mu\text{g}/\text{mL}$ OX-LDL; however, OX-LDL-induced superoxide production was markedly reduced by co-treatment of the cells with equol or NAC.

eNOS-Dependent NO Production in Response to OX-LDL

Real-time quantitative RT-PCR analysis revealed that the expression of eNOS mRNA was reduced to 56% of the control level by OX-LDL treatment. Co-treatment of cells with equol antagonized this effect. Treatment of cells with equol alone increased eNOS expression by approximately one third relative to control cells. This latter effect was suppressed by cotreatment of cells with equol and the anti-estrogen ICI 182,780 (**Fig. 6A**). These results suggested that equol directly affected eNOS mRNA mediated by the estrogen receptor.

We next monitored intracellular NO generation by co-culturing HUVECs in glass-bottom dishes in the presence of 10 μM DAF-2DA (**Fig. 6B**). We observed that treatment of cells with OX-LDL significantly reduced NO generation. The reduction in NO production in response to OX-LDL exposure was antagonized by co-treatment of the cells with equol, whereas the exposure of cells to equol alone did not significantly affect NO production ($p=0.07$) (**Fig. 6B**).

Endothelial Uptake of OX-LDL

We next evaluated the effect of equol on the cellular localization of Dil-labeled OX-LDL. HUVECs were pretreated with equol (10 μM) or a monoclonal antibody specific to LOX-1 (neutralizing anti-LOX-1 mAb inhibits Hsp70 binding to dendritic cells and Hsp70-induced antigen cross-presentation; 1:50) prior to incubation with Dil-labeled OX-LDL for 4 h. We observed striking cytoplasmic staining in Dil-OX-LDL-exposed cells, indicating cytoplasmic uptake of oxidized LDL (**Fig. 7**). Pretreatment of cells with either equol or a monoclonal antibody specific to

Fig. 6. OX-LDL-induced eNOS-dependent NO production.

A: eNOS mRNA was examined by real-time quantitative RT-PCR. Values represent the mean $\pm$ S.D. of data from three independent experiments. * $p < 0.05$ or ** $p < 0.01$. B: OX-LDL-induced intracellular NO production. Values represent the mean $\pm$ S.D. of data from three independent experiments. * $p < 0.05$.

LOX-1 inhibited the uptake of Dil-labeled OX-LDL. Microphotographs are representative of five individual slides for each experimental condition.

Discussion

We have shown that equol inhibits the induction of apoptosis in response to the treatment of cells with OX-LDL, and this effect is accompanied by the regulation of intracellular reactive oxygen species (superoxide and NO). Furthermore, equol inhibited the uptake of OX-LDL. The apoptosis of vascular cells plays a role in the progression of atherosclerotic lesions¹⁸⁻²¹. We have shown here that exposure of HUVECs to native LDL for 48 h did not lead to DNA fragmentation; however, oxidation of LDL with copper (II) prior to exposure of the HUVECs led to significant DNA laddering. These findings suggest that the prevention of LDL oxidation and suppression of OX-LDL-induced cellular effects can have a beneficial effect upon the progression of endothelial injury. The pathophysiological effects of OX-LDL on vascular cells have been well documented; recently, studies have reported that various components in foods can attenuate lipid oxidation²²⁻²⁵.

Fig. 7. Endothelial uptake of OX-LDL in HUVECs.

HUVECs were treated with Dil-labelled OX-LDL for 4 h in the presence or absence of equol (10 μ M) or a monoclonal antibody specific for LOX-1, and Dil distribution in the cytoplasm due to uptake of oxidized LDL was monitored by confocal laser scanning microscopy. Microphotographs are representative of five individual dishes.

Dietary isoflavones have been suggested to be protective against a range of diseases, including cardiovascular diseases, osteoporosis, and cancer, and have also been reported to alleviate menopausal symptoms in postmenopausal women²⁶⁻²⁸. Isoflavones (members of the class of flavonoids) belong to a group of plant compounds called phytoestrogens. Because isoflavones bear close structural similarity to 17 β -estradiol, they exhibit some degree of binding affinity for the estrogen receptor (ER α , β). ER α and ER β are both expressed throughout the vasculature²⁹. A few studies have investigated the effects of isoflavones on various circulating biomarkers of endothelial function, with beneficial effects reported on plasminogen activator inhibitor-1, endothelin-1, VCAM-1 and NO metabolites³⁰⁻³². These effects are suggested to be mediated at least partly by binding of the isoflavones to the ER. Furthermore, the concentration of isoflavones and their metabolites in plasma was monitored by gas chromatography-mass spectrometry following ingestion of soybean products. The peak plasma concentration of equol after ingestion of 60 g kinako appeared to be several hundred nanomoles³³. In this study, we examined the additional effect of equol on OX-LDL-induced apoptosis, and whether there was any contribution of

the uptake of OX-LDL in endothelial cells, to this process. To reflect the concentration of blood equol, we used equol at one thousand nanomoles or at ten-fold concentration.

We first showed that equol or the antioxidant NAC suppressed OX-LDL-stimulated apoptosis, whereas co-treatment of cells with L-NAME and equol restored the induction of apoptosis by OX-LDL (Fig. 4). These results suggest that free radicals, including superoxide and NO, may at least in part mediate OX-LDL-stimulated cell apoptosis. We further examined the effect of equol on the production of superoxide and NO in response to cell exposure to OX-LDL. We found that equol antagonized the stimulatory effect of OX-LDL on the expression of NAD(P)H oxidase subunits and led to decreased superoxide production in response to OX-LDL, whereas exposure of cells to equol alone had no effect upon the expression of NAD(P)H oxidase subunits (Fig. 5A, B, C). In addition, equol antagonized the reduction in eNOS expression and NO production in response to OX-LDL, and treatment of cells with equol alone increased eNOS expression (Fig. 6A, B). NO exhibits antiapoptotic properties, and is a highly sensitive regulator of cellular signaling processes³⁴. Taken together, these data

Fig. 8. Schematic illustration shows the effects of equol on OX-LDL-stimulated apoptosis in HUVECs.

suggest that equol may control the generation of free radicals, such as superoxide, through NO production.

We hypothesized that the effect of equol on OX-LDL-induced cell apoptosis is mediated by OX-LDL-uptake in endothelial cells. To examine this hypothesis, we evaluated the uptake of fluorescent Dil-labelled OX-LDL in HUVECs. We demonstrated that equol inhibited endothelial uptake of OX-LDL. Our data also indicated that the suppression of LOX-1 activity, a major scavenger receptor expressed in endothelial cells, inhibited endothelial uptake of OX-LDL (**Fig. 7**); however, incubation (experimental time, 4 h) of cells with OX-LDL or equol alone had no effect upon the expression of scavenger receptor (LOX-1, CD36, and so on) mRNA expression (data not shown). Although Jeong *et al.*³⁵⁾ reported that OX-LDL led to the rapid induction of LOX-1 expression that peaked at 4 h post-treatment and then rapidly declined, we did not observe a similar increase under our experimental conditions. We assume that the mechanism behind the inhibition of endothelial uptake of OX-LDL by equol is linked to the latter's antioxidant activity. On the other hand, it has been reported that the antioxidant vitamin E suppressed LOX-1-ligand binding ability and reduced atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits³⁶⁾. These results suggest that equol, which is a strong antioxidant, may also inhibit endothelial uptake of OX-LDL; however,

this effect deserves further study.

In conclusion, we show here that equol, the major metabolite of daidzein, controls intracellular superoxide and NO production, resulting in reduced cell apoptosis by OX-LDL in HUVECs (**Fig. 8**). It is suggested that the intake of soy products may be implicated in the pathogenesis of progressive atherosclerosis. Individuals who can produce equol may receive positive health benefits from soy consumption.

Acknowledgments

This study was partly supported by the Project in Nagano prefectural government.

References

- 1) Dimmeler S, Hermann C, Galle J, Zeiher AM: Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler Thromb Vasc Biol*, 1999; 19:656-664
- 2) Fujita H, Morita I, Murota S: Hydrogen peroxide induced apoptosis of endothelial cells concomitantly with cycloheximide. *J Atheroscler Thromb*, 2000; 7:209-215
- 3) Cominacini L, Rigoni A, Pasini AF, Garbin U, Davoli A, Campagnola M, Pastorino AM, Lo Cascio V, Sawamura T: The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentra-

- tion of nitric oxide in endothelial cells through an increased production of superoxide. *J Biol Chem*, 2001; 276:13750-13755
- 4) Smirnova IV, Sawamura T, Goligorsky MS: Upregulation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in endothelial cells by nitric oxide deficiency. *Am J Physiol Renal Physiol*, 2004; 287:F25-F32
 - 5) Jeong YJ, Choi YJ, Choi JS, Kwon HM, Kang SW, Bae JY, Lee SS, Kang JS, Han SJ, Kang YH: Attenuation of monocyte adhesion and oxidised LDL uptake in luteolin-treated human endothelial cells exposed to oxidised LDL. *Br J Nutr*, 2007; 97:447-457
 - 6) Liochev SI, Fridovich I: Copper, zinc superoxide dismutase and H₂O₂. Effects of bicarbonate on inactivation and oxidations of NADPH and urate, and on consumption of H₂O₂. *J Biol Chem*, 2002; 277:34674-34678
 - 7) Shin HK, Kim YK, Kim KY, Lee JH, Hong KW: Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol. *Circulation*, 2004; 109:1022-1028
 - 8) Ambra R, Rimbach G, de Pascual Teresa S, Fuchs D, Wenzel U, Daniel H, Virgili F: Genistein affects the expression of genes involved in blood pressure regulation and angiogenesis in primary human endothelial cells. *Nutr Metab Cardiovasc Dis*, 2006; 16:35-43
 - 9) Hall WL, Formanuk NL, Harnpanich D, Cheung M, Talbot D, Chowienzyk PJ, Sanders TA: A meal enriched with soy isoflavones increases nitric oxide-mediated vasodilation in healthy postmenopausal women. *J Nutr*, 2008; 138:1288-1292
 - 10) Nasca MM, Zhou JR, Welty FK: Effect of soy nuts on adhesion molecules and markers of inflammation in hypertensive and normotensive postmenopausal women. *Am J Cardiol*, 2008; 102:84-86
 - 11) Yu JY, Lee JJ, Lim Y, Kim TJ, Jin YR, Sheen YY, Yun YP: Genistein inhibits rat aortic smooth muscle cell proliferation through the induction of p27kip1. *J Pharmacol Sci*, 2008; 107:90-98
 - 12) Xiao CW: Health effects of soy protein and isoflavones in humans. *J Nutr*, 2008; 138:1244S-1249S
 - 13) Setchell KD, Brown NM, Lydeking-Olsen E: The clinical importance of the metabolite equol—a clue to the effectiveness of soy and its isoflavones. *J Nutr*, 2002; 132:3577-3584
 - 14) Jackman KA, Woodman OL, Sobey CG: Isoflavones, equol and cardiovascular disease: pharmacological and therapeutic insights. *Curr Med Chem*, 2007; 14:2824-2830
 - 15) Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, Beaudrie K, Cone JT: Single vertical spin density gradient ultracentrifugation. *Methods Enzymol*, 1986; 128:181-209
 - 16) Roberta R, Jean-Marc Z, Angelo A: Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells. *Circulation*, 2000; 102:82-87
 - 17) Delneste Y, Magistrelli G, Gaucha J, Haeuw J, Aubry J, Nakamura K, Kawakami-Honda N, Goetsch L, Sawamura T, Bonnefoy J, Jeannin P: Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunology*, 2002; 17:353-362
 - 18) Karsan A, Harlan JM: Modulation of endothelial cell apoptosis: mechanisms and pathophysiological roles. *J Atheroscler Thromb*, 1996; 3:75-80
 - 19) Nakazawa T, Xui N, Hesong Z, Kinoshita M, Chiba T, Kaneko E, Yui K, Shimokado K: Danshen Inhibits oxysterol-induced endothelial cell apoptosis in vivo. 2005; 12:132-137
 - 20) Ishino S, Mukai T, Kume N, Asano D, Ogawa M, Kuge Y, Minami M, Kita T, Shiomi M, Saji H: Lectin-like oxidized LDL receptor-1 (LOX-1) expression is associated with atherosclerotic plaque instability—analysis in hypercholesterolemic rabbits. *Atherosclerosis*, 2007; 195:48-56
 - 21) Tabas I: Apoptosis and efferocytosis in mouse models of atherosclerosis. *Curr Drug Targets*, 2007; 8:1288-1296
 - 22) Iwamoto T, Hosoda K, Hirano R, Kurata H, Matsumoto A, Miki W, Kamiyama M, Itakura H, Yamamoto S, Kondo K: Inhibition of low-density lipoprotein oxidation by astaxanthin. *J Atheroscler Thromb*, 2000; 7:216-222
 - 23) Mursu J, Voutilainen S, Nurmi T, Helleranta M, Rissanen TH, Nurmi A, Kaikkonen J, Porkkala-Sarataho E, Nyyssönen K, Virtanen JK, Salonen R, Salonen JT: Polyphenol-rich phloem enhances the resistance of total serum lipids to oxidation in men. *J Agric Food Chem*, 2005; 53:3017-3022
 - 24) Zang M, Xu S, Maitland-Toolan KA, Zuccollo A, Hou X, Jiang B, Wierzbicki M, Verbeuren TJ, Cohen RA: Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. *Diabetes*, 2006; 55:2180-2191
 - 25) Steffen Y, Jung T, Klotz LO, Schewe T, Grune T, Sies H: Protein modification elicited by oxidized low-density lipoprotein (LDL) in endothelial cells: protection by (-)-epicatechin. *Free Radic Biol Med*, 2007; 42:955-970
 - 26) Nasca MM, Zhou JR, Welty FK: Effect of soy nuts on adhesion molecules and markers of inflammation in hypertensive and normotensive postmenopausal women. *Am J Cardiol*, 2008; 102:84-86
 - 27) Wu AH, Koh WP, Wang R, Lee HP, Yu MC: Soy intake and breast cancer risk in Singapore Chinese Health Study. *Br J Cancer*, 2008; 99:196-200
 - 28) Ma DF, Qin LQ, Wang PY, Katoh R: Soy isoflavone intake increases bone mineral density in the spine of menopausal women: meta-analysis of randomized controlled trials. *Clin Nutr*, 2008; 27:57-64
 - 29) Register TC, Adams MR: Coronary artery and cultured aortic smooth muscle cells express mRNA for both the classical estrogen receptor and the newly described estrogen receptor beta. *J Steroid Biochem Mol Biol*, 1998; 64:187-191
 - 30) Rios DR, Rodrigues ET, Cardoso AP, Montes MB, Franceschini SA, Toloi MR: Effects of isoflavones on the coagulation and fibrinolytic system of postmenopausal women. *Nutrition*, 2008; 24:120-126
 - 31) Hall WL, Vafeiadou K, Hallund J, Bügel S, Koebnick C, Reimann M, Ferrari M, Branca F, Talbot D, Dadd T, Nilsson M, Dahlman-Wright K, Gustafsson JA, Miniñane AM, Williams CM: Soy-isoflavone-enriched foods and

- inflammatory biomarkers of cardiovascular disease risk in postmenopausal women: interactions with genotype and equol production. *Am J Clin Nutr*, 2005; 82:1260-1268
- 32) Squadrito F, Altavilla D, Morabito N, Crisafulli A, D'Anna R, Corrado F, Ruggeri P, Campo GM, Calapai G, Caputi AP, Squadrito G: The effect of the phytoestrogen genistein on plasma nitric oxide concentrations, endothelin-1 levels and endothelium dependent vasodilation in postmenopausal women. *Atherosclerosis*, 2002; 163:339-347
- 33) Watanabe S, Yamaguchi M, Sobue T, Takahashi T, Miura T, Arai Y, Mazur W, Wähälä K, Adlercreutz H: Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J Nutr*, 1998; 128:1710-1715
- 34) Kotamraju S, Hogg N, Joseph J, Keefer LK, Kalyanaraman B: Inhibition of oxidized low-density lipoprotein-induced apoptosis in endothelial cells by nitric oxide. Peroxyl radical scavenging as an antiapoptotic mechanism. *J Biol Chem*, 2001; 276:17316-17323
- 35) Jeong YJ, Choi YJ, Choi JS, Kwon HM, Kang SW, Bae JY, Lee SS, Kang JS, Han SJ, Kang YH: Attenuation of monocyte adhesion and oxidised LDL uptake in luteolin-treated human endothelial cells exposed to oxidised LDL. *Br J Nutr*, 2007; 97:447-457
- 36) Oka K, Yasuhara M, Suzumura K, Tanaka K, Sawamura T: Antioxidants suppress plasma levels of lectinlike oxidized low-density lipoprotein receptor-ligands and reduce atherosclerosis in Watanabe heritable hyperlipidemic rabbits. *J Cardiovasc Pharmacol*, 2006; 48:177-183