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Oligomerized grape seed polyphenols attenuate inflammatory changes due to antioxidative properties in coculture of adipocytes and macrophages

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Abstract

Macrophage infiltration of white adipose tissue (WAT) is implicated in the metabolic complications of obesity. In addition, inflammatory changes through dysregulated expression of inflammation-related adipokines such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) in WAT are considered to be one of the causes of insulin resistance. Recently, enhanced oxidative stress in adipocytes has been reported to be implicated in dysregulated expression of inflammation-related adipokines. Polyphenols are well known as potent natural antioxidants in the diet. In the present study, we investigated the antioxidative effects of an oligomerized grape seed polyphenol (OGSP) on inflammatory changes in coculture of adipocytes and macrophages. Coculture of HW mouse white adipocytes and RAW264 mouse macrophages markedly increased the production of TNF-α, MCP-1 and plasminogen activator inhibitor-1 compared with control culture. Treatment of HW cells with OGSP significantly attenuated the dysregulated production of adipokines. Moreover, OGSP significantly suppressed coculture-induced production of reactive oxygen species (ROS). Although enhanced release of free fatty acids (FFAs) by coculture was not altered by OGSP, FFA-induced ROS production in HW cells was significantly attenuated by OGSP. Furthermore, OGSP significantly reduced increases in the transcriptional activity of nuclear factor-κB and activation of extracellular signal-regulated kinase by coculture. Thus, these results suggest that the antioxidative properties of OGSP attenuate inflammatory changes induced by the coculture of adipocytes and macrophages.

Keywords: Oligomerized grape seed polyphenol; Adipocytes; Anti-inflammatory effects; Antioxidative effects; Adipokines

1. Introduction

Obesity is associated with numerous diseases, including diabetes and cardiovascular disease [1]. Recently, it has been suggested that obesity is associated with chronic inflammatory response [2,3]. Indeed, an increased number of infiltrating macrophages and dysregulated secretion of some inflammation-related adipokines has been observed in the white adipose tissue (WAT) of obese subjects [4–6]. For example, expression levels of the pro-inflammatory adipokines tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) are up-regulated in the WAT of obese mice [5,7,8]. Overexpression of MCP-1 in adipocytes promotes macrophage infiltration in WAT, and exposure of adipocytes to TNF-α and MCP-1 results in blockage of insulin-induced glucose uptake in adipocytes [8–10]. Moreover, in addition to impairing insulin signaling, it has been reported that TNF-α regulates expression of other adipokines, such as MCP-1 and plasminogen activator inhibitor-1 (PAI-1), in adipocytes [10,11]. TNF-α up-regulates PAI-1 expression in adipocytes via the pathway that involves activation of signaling molecules, such as extracellular signal-regulated kinase (ERK), protein kinase C

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and nuclear factor-κB (NF-κB) [11]. The level of PAI-1 in plasma is increased in obese patients, and many reports have shown that increased PAI-1 levels contribute to the development of obesity [12]. These reports indicate that the inflammatory response in WAT is one of the potential mechanisms of obesity-induced insulin resistance.

A previous report has demonstrated that adipocytes generate reactive oxygen species (ROS) and that the levels of oxidative stress are increased in the WAT of KKAy mice, which act as a diabetic model [13]. Increased oxidative stress in adipocytes causes dysregulated expression of adipokines, including MCP-1 and PAI-1 [13–15]. Moreover, increased oxidative stress in adipocytes has been found to impair insulin signaling [16]. In fact, prolonged exposure of 3T3-L1 adipocytes to micromolar concentrations of H_2O_2 inhibits insulin-induced translocation of glucose transporter 4 to the plasma membrane, thereby inhibiting glucose uptake [17].

Grapes are one of the most widely consumed fruits in the world. Grape seeds are rich in polyphenols, including catechin, epicatechin and procyanidin [18]. One of the characteristics of grape seed polyphenols (GSPs) is that they are mixtures of monomers, dimers and oligomers of catechin and/or epicatechin, while tea polyphenols are only monomers of catechin and epicatechins with gallate substitution [18–20]. In general, it is thought that high-molecular-weight forms of polyphenols are more difficult to absorb than those with low molecular weights. Actually, Fujii et al. [21] have found that oligomerized forms of purified GSPs are more conducive to higher absorption rates in vivo than non-oligomerized forms. In addition, recent studies have shown that procyanidin in grape seeds possesses antioxidative and anti-inflammatory activities; for example, grape seed procyanidin acts as an anti-inflammatory agent in endotoxin-stimulated RAW264 macrophages by inhibiting NF-κB [19].

In the present study, we hypothesized a possibility that oxidative stress is implicated in inflammatory response of adipocytes and that polyphenols may possess anti-inflammatory effects through their antioxidative effects. Thus, we investigated the antioxidative effects of oligomerized grape seed polyphenols (OGSPs) developed by our group [21,22] on inflammatory changes in coculture of adipocytes and macrophages.

2. Materials and methods

2.1. Oligomerized grape seed polyphenol

OGSP was obtained by oligomerizing the purified GSP polymers using a modification of a patented technology previously described [21]. In brief, the process involves the eluate extraction of powdered dried fruits with 80% (v/v) methanol. The filtrate is subjected to a DAIMON HP-20 column, and after washing with H_2O, the eluate is evaporated to dryness, yielding a dark brown powder consisting of a mixture of proanthocyanidins. The resulting mixture is combined with L-cysteine hydrochloride monohydrate and L-ascorbic acid in H_2O and heated at 60°C for 48 h. The reaction mixture is filtered through a DAIMON HP-20 column, washed with H_2O and eluted with 40% (v/v) ethanol. Evaporation of the eluate yields a reddish brown powder, the oligomeric proanthocyanidin–cysteine complexes. The structure of the major components of OGSP was confirmed, by analysis of mass and nuclear magnetic resonance spectra, to be 4-*S*-cysteine derivatives of procyanidins B-1 and B-2, that is, epicatechin-(4β-8)-epicatechin-(4β-8)-catechin and epicatechin-(4β-8)-epicatechin-(4β-8)-epicatechin, respectively.

2.2. Cell culture

HW mouse white preadipocytes were kindly provided by Professor M. Saito (Tenshi University, Sapporo, Japan) [23]. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Differentiation to adipocytes was induced by treatment with 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine for 48 h. The treated cells were maintained in DMEM containing 10 μg/ml of insulin and 50 nM 3-3′-5-triod-L-thyronine for 72 h to accumulate the triglyceride content. Fully differentiated cells were treated with OGSP (10 and 20 μg/ml) for 24 h. The murine macrophage cell line RAW264 (RCB0535) was purchased from RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in DMEM supplemented with 10% FCS.

2.3. Cytotoxicity assay

The cytotoxic activity of OGSP for HW cells was determined by measuring the level of lactate dehydrogenase (LDH) in culture medium released from HW cells. The level of LDH was measured by using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol.

2.4. Measurement of ROS production in adipocytes

Measurement of ROS production in the HW cells was performed as described previously [13]. ROS production was measured by nitroblue tetrazolium (NBT) reduction. HW cells were incubated for 60 min in PBS (137 mM NaCl, 8.1 mM Na_2PO_4, 2.68 mM KCl and 1.47 mM KH_2PO_4) containing 0.2% NBT. Formazan was dissolved in 50% acetic acid, and the absorbance was monitored at 560 nm using a spectrophotometer.

2.5. Measurement of inflammation-related adipokines in culture medium

The levels of TNF-α, MCP-1 and PAI-1 in culture medium were measured using a Mouse TNF-α ELISA Kit (BioSource, Nivelles, Belgium), Mouse MCP-1 ELISA (Bender Medsystems, Burlingame, CA, USA) and Mouse PAI-1 Total Antigen Kit (Innovative Research, Peary Court Novi, MI, USA), respectively.

2.6. Coculture of adipocytes and macrophages

Adipocytes and macrophages were cocultured in a contact system, as previously described [24]. Serum-starved fully differentiated HW cells were cultured in a 60-mm dish, and RAW264 cells (3×10^5 cells) were plated onto HW cells for 24 h. HW cells were pretreated with vehicle or OGSP (10 and 20 μg/ml) for 24 h. As a control, equal numbers of both cells were cultured separately and mixed after 24 h. After coculture, the supernatant was collected and stored at −80°C until levels of inflammation-related adipokines and ROS in both cells were measured by NBT assay, as described above.

2.7. Lipolysis assay

The levels of free fatty acid (FFA) in coculture medium were measured as described elsewhere [24]. Fully differentiated HW cells were cultured with vehicle or OGSP (10 and 20 μg/ml) for 24 h and cocultured with RAW264 cells in the medium containing 2% FFA-free bovine serum albumin (BSA) (Sigma Chemical, St. Louis, MO, USA). The concentration of FFAs in the medium was measured using an acyl-coenzyme A oxidase-based colorimetric assay kit (NEFA-C, WAKO, Osaka, Japan).

2.8. FFA treatment

Fully differentiated HW cells were cultured with vehicle or OGSP (10 and 20 μg/ml) for 24 h and incubated with or without 300 μM linoleic acid (Sigma) in serum-free DMEM containing 2% FFA-free BSA for 24 h. ROS in these cells were measured by NBT assay as described above.

2.9. Luciferase assay

pNF-κB-Luc vector (1 μg) (BD Bioscience, San Jose, CA, USA) was transfected into HW cells cultured in 24-well plates with Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The cell cultures were differentiated 24 h after transfection, as described above. The differentiated HW cells were treated with vehicle or OGSP (10 and 20 μg/ml) for 24 h and then cocultured with RAW264 cells (3×10^4 cells) for 24 h. Cell extracts for luciferase assay were prepared with the Cell Culture Lysis Reagent (Promega, Madison, WI, USA). Luciferase activity was assayed with Luciferse Assay Systems (Promega) using a luminometer.

2.10. Preparation of protein extracts and Western blot analysis

Total protein was extracted with Tissue Protein Extraction Reagent (PIERCE, Rockford, IL, USA) containing 50 mM sodium fluoride, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/ml of aprotinin and 5 mg/ml of leupeptin. Each protein sample was subjected to 10–12% NuPAGE (Invitrogen) gradient gel electrophoresis and transferred onto a nylon membrane. The membrane was blocked using TBS-T (20 mmol/L of Tris–HCl, pH 7.5, 137 mM of NaCl and 0.1% Tween-20) containing 5% nonfat dry milk and then probed with an anti-phosphorylated ERK1/2 antibody (sc-16982-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or non-phosphorylated anti-ERK1/2 antibody (Promega). After washing with TBS-T, bound antibody was detected using the ECL system (Amersham, Buckinghamshire, UK). The intensities of bands from Western blot analyses were quantified with the National Institute of Health Image Computer program.

2.11. Statistical methods

Values represent mean±S.E. Analysis of variance was used to compare the values, and the Tukey–Kramer method was used for the post hoc test. Differences were considered significant at $P<.05$.

3. Results

3.1. Effect of OGSP on cytotoxicity and ROS production in adipocytes

We first investigated the cytotoxicity and antioxidative effects of OGSP on adipocytes by measuring LDH released from damaged cells and NBT reduction, respectively. No cytotoxicity of OGSP was observed when fully differentiated HW cells were treated with OGSP, whereas LDH release increased markedly when HW cells were cultured under culture medium containing 1% Triton X, which was used as positive control (Fig. 1A). On the other hand, OGSP decreased the levels of ROS in fully differentiated HW cells in a dose-dependent manner (Fig. 1B). These results suggest that decrease of oxidative stress in adipocytes by OGSP is not due to cytotoxicity but due to antioxidative effects.

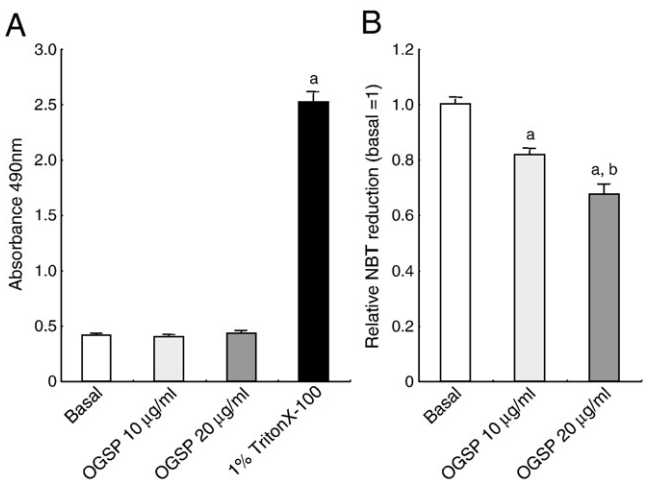

Fig. 1. Effect of OGSP on production of ROS in HW cells. Fully differentiated HW cells were treated with 10 and 20 μg/ml of OGSP for 24 h. (A) Cytotoxicity of OGSP for HW cells was assessed by measuring LDH released from these cells. (B) ROS production was measured by NBT reduction. The data are ratios, with the basal value=1 and expressed as mean±S.E. (n=3). [a]$P<.05$ versus basal; [b]$P<.05$ versus OGSP 10 μg/ml.

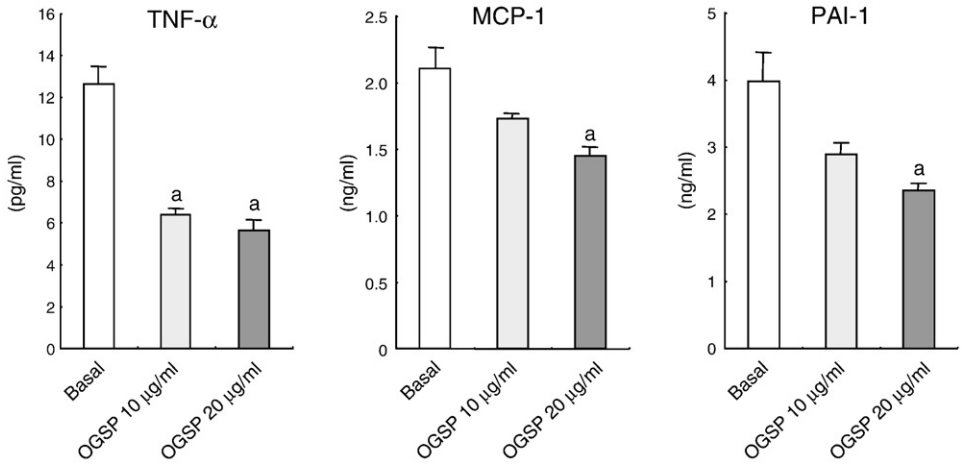

Fig. 2. Effect of OGSP on production of inflammation-related adipokines in HW cells. Fully differentiated HW cells were treated with 10 and 20 μg/ml of OGSP for 24 h. The levels of TNF-α, MCP-1 and PAI-1 in culture medium were measured by ELISA. The mean±S.E. (n=3) is given. [a]P<.05 versus basal.

3.2. Effect of OGSP on inflammation-related adipokine production in adipocytes

Subsequently, the effects of OGSP on secretion of inflammation-related adipokines from HW cells were examined with an ELISA. As shown in Fig. 2, the levels of TNF-α proteins in culture medium containing 10 and 20 μg/ml of OGSP were significantly lower than those of the basal condition. Meanwhile, MCP-1 and PAI-1 secretion in the culture medium was significantly decreased by treatment with 20 μg/ml OGSP for 24 h but not by treatment with 10 μg/ml OGSP (Fig. 2).

3.3. Effect of OGSP on inflammatory changes by coculture of adipocytes and macrophages

Fig. 2 indicates that OGSP could decrease the expression of pro-inflammatory adipokines, such as TNF-α and MCP-1, in adipocytes. Thus, we investigated the effect of OGSP on inflammatory changes in coculture of adipocytes and macrophages. Fully differentiated HW cells were treated with OGSP (10 and 20 μg/ml) for 24 h and then cocultured with RAW264 cells in contact systems for 24 h [24]. Coculture of HW and RAW264 cells up-regulated secretion of TNF-α, MCP-1 and PAI-1, compared with the control culture (Fig. 3). Although treatment of HW cells with 10 μg/ml OGSP did not influence enhanced secretion of those adipokines, treatment with 20 μg/ml OGSP significantly reduced secretion of TNF-α, MCP-1 and PAI-1 in coculture (Fig. 3).

3.4. Effect of OGSP on increases of oxidative stress in coculture of adipocytes and macrophages

We next investigated whether oxidative stress is affected by coculture of adipocytes and macrophages. As shown in Fig. 4A, the levels of ROS in coculture condition were higher than those in the control culture, and 20 μg/ml OGSP treatment of HW cells definitely attenuated such coculture-induced increases of ROS, although 10 μg/ml OGSP treatment did not attenuate it. On the other hand, although release of FFAs, which increase ROS production in

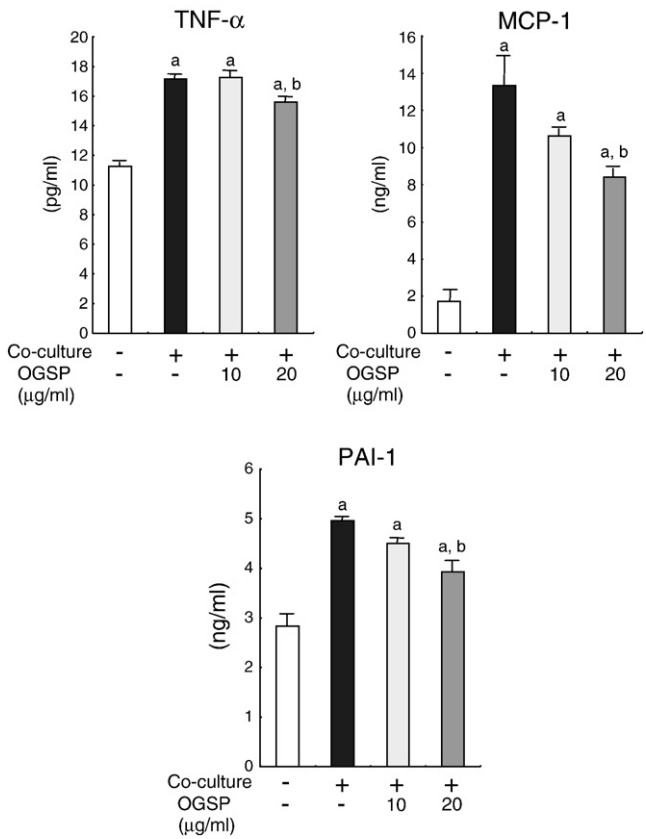

Fig. 3. Effects of OGSP on inflammatory changes in coculture of HW and RAW264 cells. Fully differentiated HW cells were treated with 10 and 20 μg/ml of OGSP for 24 h, and OGSP-treated cells were cocultured with RAW264 cells (3×10^5 cells/well) for 24 h. The levels of TNF-α, MCP-1 and PAI-1 in culture medium were measured by ELISA. The mean±S.E. (n=3) is given. [a]P<.05 versus control culture; [b]P<.05 versus coculture without OGSP condition.

adipocytes [13,25], was significantly increased in coculture, as reported previously [24], OGSP did not affect coculture-induced increases of FFAs (Fig. 4B). However, treatment with 20 μg/ml OGSP significantly attenuated linoleic-acid-induced increases of ROS production in HW cells (Fig. 4C).

3.5. Effect of OGSP on the transcriptional activity of NF-κB and the activation of ERKs under inflammatory changes

Transcription factor NF-κB has been found to be activated by oxidative stress in various cells and to regulate the expression of genes for TNF-α and MCP-1 [26–28]. On the other hand, activation of ERKs is also regulated by oxidative stress and is correlated with the expression of the gene for MCP-1 [29,30]. Hence, to identify the mediators of the anti-inflammatory effects of OGSP, we measured the transcriptional activity of NF-κB in HW cells and the activation of ERKs in coculture. Consistent with the previous study [27], the transcriptional activity of NF-κB in HW cells was markedly increased in coculture with RAW246 cells (Fig. 5A). In addition, coculture-induced activation of NF-κB was significantly attenuated by treatment of HW cells with 20 μg/ml OGSP (Fig. 5A). Moreover, phosphorylation of ERK1 (p44) and ERK2 (p42) was enhanced in coculture, but treatment of HW cells with 20 μg/ml OGSP significantly attenuated coculture-induced phosphorylation of ERKs (Fig. 5B and C), although treatment with 10 μg/ml OGSP did not alter it.

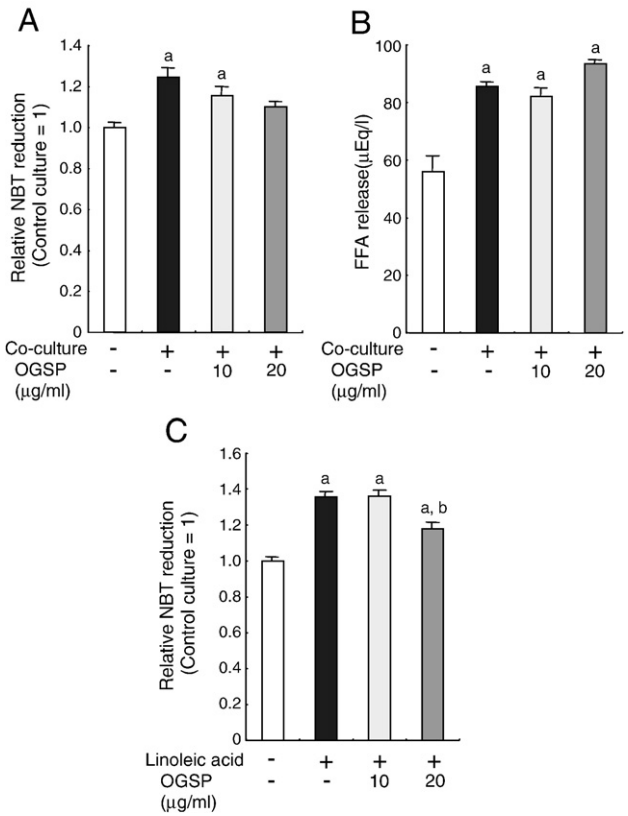

Fig. 4. Effects of OGSP on the levels of ROS and FFA release in inflammatory changes. Fully differentiated HW cells were treated with 10 and 20 μg/ml of OGSP for 24 h, and OGSP-treated cells were cocultured with RAW264 cells (3×10^5 cells/dish) for 24 h. (A) ROS production was measured by NBT reduction. The data were expressed as ratios with the value of the control culture=1. (B) FFA release from coculture was measured by acyl-coenzyme A oxidase-based colorimetric assay. (C) Fully differentiated HW cells were treated with 300 μM linoleic acid for 24 h. HW cells were pretreated with 10 and 20 μg/ml of OGSP for 24 h prior to treatment with linoleic acid. ROS production was measured by NBT reduction. The data are expressed as ratios with the value of the control culture=1. The mean±S.E. (n=3) is given. [a]P<.05 versus control culture; [b]P<.05 versus linoleic acid treatment only.

4. Discussion

In the present study, we have demonstrated that OGSP attenuates not only inflammatory changes in coculture of adipocytes and macrophages but also coculture-induced ROS production. Because oxidative stress in the WAT of diabetic KKAy mice is significantly higher than that of control C57BL/6 mice, and dysregulated expression of genes for adipokines, including MCP-1 and PAI-1, has been observed in 3T3-L1 adipocytes exposed to oxidative stress agents such as H_2O_2 [13–15], increased oxidative stress might be one of the reasons for dysregulated expression of inflammation-related adipokines induced by coculture.

FFAs are released by lipolysis of triglycerides in adipocytes and exhibit various biological effects, involving insulin resistance in adipocytes [31]. Current reports demonstrate that release of FFAs in coculture induces inflammatory changes, such as increased production of TNF-α through Toll-like receptor-4 in macrophages [24,27]. In addition, it has been reported that TNF-α induces lipolysis and FFA release in adipocytes [32,33]. From the finding that the positive correlation was observed between ROS production and release of FFAs in coculture (Fig. 4A and B) and that treatment of FFAs resulted in increased ROS production in adipocytes (Fig. 4C), it might be speculated that coculture-derived release of FFAs enhanced ROS production in adipocytes, although it is difficult to determine whether ROS production increases in adipocytes and/or in macrophages. However, treatment with 20 μg/ml OGSP did not affect the release of FFAs in coculture (Fig. 4B), although it significantly reduced secretion of both TNF-α and ROS production (Figs. 3 and 4A). The reason for this discrepancy might be due to the lipolytic effects of polyphenols present in adipocytes. In fact, Ardevol et al. [34] have reported that grape seed procyanidins stimulate lipolysis in 3T3-L1 adipocytes. Thus, the levels of FFAs in coculture without OGSP and coculture with 20 μg/ml OGSP showed similar results. On the other hand, increases in ROS production induced by the FFA linoleic acid were significantly attenuated by 20 μg/ml OGSP (Fig. 4C). This result suggested that the antioxidative effects of OGSP on the inflammatory changes in coculture were not due to a decrease in FFAs but rather due to the suppression of FFA-induced production of ROS in adipocytes. However,

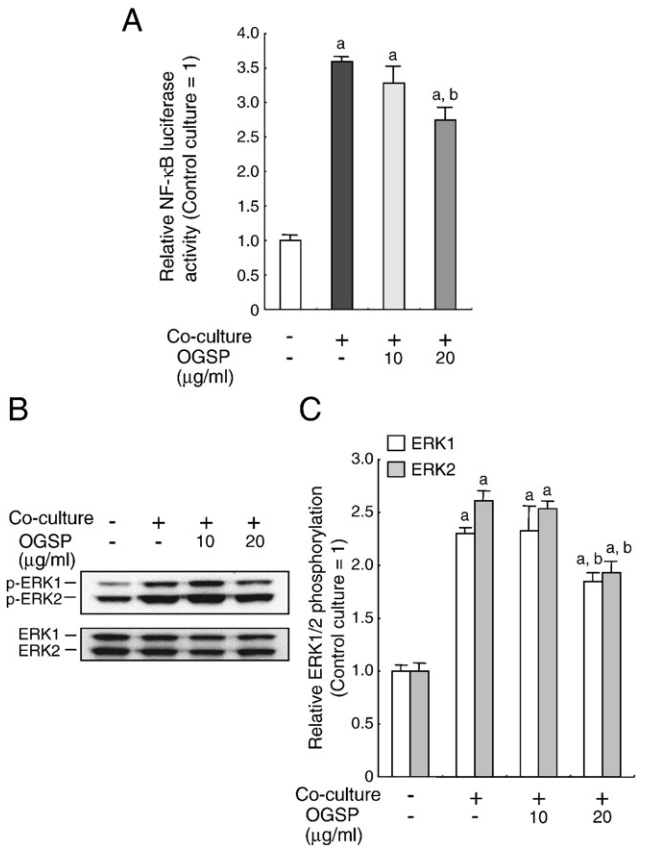

Fig. 5. Effects of OGSP on transcriptional activity of NF-κB and phosphorylation of ERK1/2 in inflammatory changes. Transcriptional activity of NF-κB (A). HW cells were transiently transfected with the pNF-κB-Luc vector before differentiation was induced. The transfected cells were treated with 10 and 20 μg/ml of OGSP for 24 h after differentiation and then cocultured with RAW264 cells (3×10^4 cells/well) for 24 h. Transcriptional activity of NF-κB was measured with a luminometer. Phosphorylation of ERK1/2 (B and C). Total protein (10 μg) was extracted from each culture and then subjected to Western blot analysis. Representative data from Western blot analyses are shown in Panel B. Phosphorylation level of ERK1/2 was normalized to that of the expression level of ERK1/2 (C). The data were expressed as ratios with the value of the control culture=1. The mean±S.E. (n=3) is given. [a]P<.05 versus control culture; [b]P<.05 versus coculture without OGSP condition.

although treatment with linoleic and stearic acids results in increased ROS production and dysregulated expression of inflammation-related adipokines, no significant inflammatory changes in adipocytes occur when incubated with oleic acid [24,25,35]. Thus, the specific type of FFA released from adipocytes is thought to be important for the occurrence of inflammatory changes in the WAT of obese patients.

The transcription factor NF-κB and the signal transduction molecules mitogen-activated protein kinases, including ERK, c-Jun N-terminal kinase, stress-activated protein kinase and the p38 subfamilies, are activated by oxidative stress and thought to be important mediators of oxidative-stress-induced signal transduction [26,29]. *N*-Acetylcysteine, a potent antioxidant, inhibits NF-κB activation and TNF-α-induced increased expression of the PAI-1 in 3T3-L1 adipocytes, and activation of ERK is implicated in the expression of the MCP-1 [30,36]. Moreover, it has been found that NF-κB modulates expression of the genes for MCP-1 in certain cell types [27,28]. Thus, to determine the molecular mechanisms of OGSP-induced anti-inflammatory effects, we focused on NF-κB and ERKs. As expected, there appeared to be a good correlation between attenuation of coculture-induced increase in oxidative stress and activation of those molecules in adipocytes (Figs. 4A and 5A–C). These results suggest that the antioxidative mechanism of OGSP in attenuation of inflammation-related adipokines functions by suppressing the activation of NF-κB and ERKs.

Although treatment with 10 μg/ml OGSP significantly reduced ROS production in adipocytes (Fig. 1), inflammatory changes including increased ROS production were not altered in coculture of adipocytes and macrophages (Figs. 3–5). This result indicates that certain antioxidative capacity of polyphenol is necessary to suppress inflammatory changes. We have previously found that the antioxidative effects of oligomerized lychee-derived polyphenols in adipocytes are stronger than those of non-oligomerized ones, and the intensity of the antioxidative effects of these polyphenols in adipocytes is clearly reflected in the expression of genes for adipokines, including TNF-α and MCP-1 [37]. In addition, several investigators have revealed a relationship between the degree of polymerization in polyphenols and the enzyme inhibition. For instance, inhibitory effects of procyanidins on pancreatic lipase activity increased with a rise in the degree of polymerization [38]. Among procyanidins consisting of dimers, tetramers or hexamers, procyanidin tetramers exhibited the most inhibitory effect on angiotensin-I-converting enzyme activities [39]. These reports suggest that although intercellular mechanisms are still unclear, proper polymeric forms of polyphenol are different depending on their specific biological effects. Some studies, on the other hand, have proposed that the degree of polymerization of polyphenol relates to their absorption coefficient in the body. For example, it has been reported that oligomerized forms of purified GSPs are more conducive to higher absorption rates in vivo than non-oligomerized ones [21]. In addition, procyanidin dimers B2 and B5 are degraded to epicatechin in the isolated rat small intestine [40]. Because the cleavage of procyanidin dimers B2 and B5 during transfer across the small intestine seems to be energy dependent [40], if high molecular polyphenol polymers are transferred across the small intestine, considerable energy would be needed. Therefore, the absorption of polyphenol polymer might be difficult. Considering the present findings in addition to previous reports, it is possible that the degree of polymerization of polyphenol is one of the important factors involved in various biological effects of polyphenol and that as for antioxidative effects in adipocytes, oligomerized forms are more effective than non-oligomerized ones. Thus, further investigation into how much antioxidative effects and absorption coefficient of OGSP influence its anti-

inflammatory effects and its intercellular mechanisms seems to be warranted.

In conclusion, the results obtained have suggested that OGSP attenuates inflammatory changes induced by coculture of adipocytes and macrophages. One of the possible mechanisms for anti-inflammatory effects of OGSP may be suppression of inflammatory changes due to FFA-induced ROS production in adipocytes through the antioxidative effects. Thus, intake of OGSP may have beneficial effects in preventing obesity-induced metabolic syndrome.

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