RESEARCH ARTICLE

# Diosgenin attenuates inflammatory changes in the interaction between adipocytes and macrophages

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Received: May 6, 2009
Revised: June 17, 2009
Accepted: June 29, 2009

Obese adipose tissues are characterized by the enhanced infiltration of macrophages. It is considered that the paracrine loop involving monocyte chemoattractant protein-1, tumor necrosis factor-α, and the free fatty acid between adipocytes and macrophages establishes a vicious cycle that aggravates inflammatory changes and insulin resistance in obese adipose tissues. Diosgenin, a saponin aglycon found in a variety of plants, has anti-inflammatory properties. In the present study, we examined the effect of diosgenin on the inflammatory changes in the interaction between adipocytes and macrophages. A coculture of 3T3-L1 adipocytes and RAW 264 macrophages markedly enhanced the production of tumor necrosis factor-α, monocyte chemoattractant protein-1, and nitric oxide compared with the sum of their single cultures; however, treatment with diosgenin inhibited the production of these proinflammatory mediators. Diosgenin also suppressed the inflammation in RAW 264 macrophages that was induced by the conditioned medium derived from 3T3-L1 adipocytes. Furthermore, diosgenin inhibited the conditioned medium-induced degradation of inhibitor κB and the phosphorylation of c-jun N-terminal kinase in macrophages. These results indicate that diosgenin exhibits anti-inflammatory properties in the interaction of adipocytes and macrophages by inhibiting the inflammatory signals in macrophages. Diosgenin may be useful for ameliorating the inflammatory changes in obese adipose tissues.

**Keywords:**
Adipocyte / Diosgenin / Inflammation / Macrophage / Monocyte chemoattractant protein-1

## 1 Introduction

Obesity is associated with the increased occurrence of numerous diseases including insulin resistance, cardiovascular diseases, and type 2 diabetes [1]. Recent studies have indicated that obesity is characterized by low-grade chronic inflammation, suggesting that inflammation may be a potential mechanism by which obesity leads to insulin resistance [2, 3].

In addition to mature adipocytes, adipose tissues contain various cells including preadipocytes, fibroblasts, endothelial cells, and macrophages. This implies the occurrence of paracrine interactions between adipocytes and nonadipocytes. Recent studies have demonstrated that obese adipose tissue is characterized by the enhanced infiltration of macrophages [4, 5]. Macrophages are crucial contributors to inflammation stimulated through toll-like receptors (TLRs) and produce various inflammatory proteins including tumor

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**Abbreviations: AP-1,** activator protein 1; **CM,** conditioned medium; **FBS,** fetal bovine serum; **FFA,** free fatty acid; **IκB,** inhibitor κB; **JNK,** c-Jun N-terminal kinase; **MCP-1,** monocyte chemoattractant protein-1; **MAPK,** mitogen-activated protein kinase; **NF-κB,** nuclear factor-κB; **NO,** nitric acid; **TNF-α,** tumor necrosis factor-α; **TLR,** toll-like receptor

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necrosis factor (TNF)-α and nitric oxide (NO). The nuclear factor-κB (NF-κB) pathway and mitogen-activated protein kinase (MAPK) pathways play an important role in TLR-mediated inflammation [6]. TNF-α and inducible NO synthase expression levels are elevated in adipose tissues of various models of rodent and human obesity [7–9].

In obese adipose tissues, monocyte chemoattractant protein (MCP)-1, a member of the CC chemokine superfamily, is also upregulated [10], suggesting that MCP-1 plays a crucial role in adipose tissue inflammatory responses by activating and inducing the infiltration of macrophages into adipose tissues. Suganami *et al.* [11] reported that the paracrine loop involving adipocyte-derived free fatty acid (FFA), MCP-1, and macrophage-derived TNF-α establishes a vicious cycle that aggravates the inflammatory changes and insulin resistance in obese adipose tissue. Therefore, anti-inflammatory compounds may ameliorate obesity-related diseases by suppressing the expression of these proinflammatory factors in adipose tissues.

Diosgenin, a saponin aglycon, is found in a variety of plants [12, 13] including fenugreek (*Trigonella foenum graecum*), roots of wild yam (*Dioscorea villosa*), *Solanum xanthocarpum* [14], *S. incanum Lloydia* [15], and *Costus speciosus* [16]. Extracts from these plants have been traditionally used for the treatment of diabetes [17], hypercholesterolemia [18], and gastrointestinal ailments [19]. Many researchers have shown that diosgenin has a large variety of biological functions, such as antifungal [20], anticancer [21], and anti-inflammatory [22] functions. In particular, the anti-inflammatory effect of diosgenin is attributed to its regulation of the activity of lipoxygenase [23], cyclooxygenase-2 [24], and NF-κB [25]. Furthermore, diosgenin has been shown to antagonize the chemokine receptor CXCR3, which mediates inflammatory responses [26].

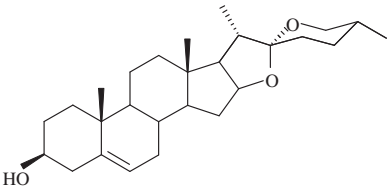
Here, we investigated the effects of diosgenin on the production of proinflammatory mediators in the interaction between adipocytes and macrophages and determined the underlying mechanism of the action.

## 2 Materials and methods

### 2.1 Chemical reagents and cells

Diosgenin (Fig. 1) was obtained from Wako (Osaka, Japan) and dissolved in ethanol. All other chemicals, guaranteed to be of reagent or tissue-culture grade, were from Sigma (MO, USA) or Nacalai Tesque (Kyoto, Japan).

RAW 264 macrophages (RIKEN BioResource Center, Tsukuba, Japan) and 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (Sigma) with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin (Gibco BRL, NY, USA) at 37°C in a humidified 5% $CO_2$ atmosphere. The differentiation of 3T3-L1 preadipocytes was induced by adipogenic agents [0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque), 0.25 μM dexamethasone (Sigma), and 10 μg/mL insulin (Sigma)] in




**diosgenin**

**Figure 1.** Chemical structure of diosgenin.

DMEM containing 10% FBS for 2 days after the cells reached confluence as described previously [27, 28]. The medium was then replaced with DMEM containing 10% FBS and 5 μg/mL insulin, and was changed every 2 days. At 20 days after the induction of differentiation, the cells that had accumulated large droplets were used as hypertrophied 3T3-L1 adipocytes. The serum-free medium of hypertrophied 3T3-L1 adipocytes cultured for 12 h was collected as a conditioned medium (CM) and stored at −20°C until use.

### 2.2 Coculture of adipocytes and macrophages

Adipocytes and macrophages were cocultured in a contact system as described previously [29, 30]. Briefly, RAW 264 cells ($3 \times 10^5$ cells/mL) were plated onto dishes with serum-starved and hypertrophied 3T3-L1 adipocytes, and the coculture was incubated in serum-free DMEM for 24 h. RAW 264 and 3T3-L1 cells of equal numbers to those in the coculture were cultured separately as control cultures. Diosgenin was added to the cultures at various concentrations, as shown in Figs. 2–4. After 24 h of treatment, the culture supernatants were collected and stored at −20°C until use. The viabilities of RAW 264 cells and 3T3-L1 cells were measured by CellTiter96 Aqueous One Solution Cell Proliferation Assay (Promega, WC, USA) after treatment of diosgenin for 24 h.

### 2.3 Measurement of TNF-α and MCP-1 production

The concentrations of TNF-α and MCP-1 in the culture supernatants were determined by ELISA, which were conducted using a READY-SET-GO! Mouse TNF-α and MCP-1 kit (eBioscience, CA, USA) in accordance with the manufacturer's instructions. The data of the ''control culture'' were calculated as the sum of the secretion levels in RAW 264 and 3T3-L1 cells single culture.

### 2.4 Measurement of NO release

The amount of nitrite in the cell-free culture supernatants was measured using Griess reagent [31]. Briefly, 100 μL

supernatant was mixed with an equivalent volume of Griess reagent (0.1% *N*-1-naphthyl-ethylenediamine in distilled water and 1% sulfanilamide in 5% phosphoric acid, 1:1) on a 96-well flat-bottom plate. After 10 min, the absorbance at 570 nm was measured, and the amount of nitrite was calculated from the $NaNO_2$ standard curve.

### 2.5 RNA preparation and real-time fluorescence-monitoring PCR

Total RNA was prepared from cultured cells using Sepasol (Nacalai Tesque) in accordance with the manufacturer's instructions. Using M-MLV reverse transcriptase (Invitrogen, CA, USA), total RNA was reverse-transcribed in accordance with the manufacturer's instructions using a thermal cycler (TaKaRa PCR Thermal Cycler SP: TaKaRa Shuzo, Shiga, Japan). To quantify mRNA expressions, real-time PCR was performed using a LightCycler System (Roche Diagnostics, Mannheim, Germany) and SYBR green fluorescence signals, as described previously [32, 33]. The real-time PCR program was 40 cycles at 95°C for 5 s, 60°C for 5 s, and 72°C for 25 s. Primers for different genes are listed in Table 1. The level of 36B4 mRNA was adopted as the internal standard for the determination of targeted mRNA levels.

### 2.6 Western blotting

Western blotting was performed as described previously [27, 29]. In brief, RAW 264 cells were washed with PBS and placed immediately in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 15 mM NaCl, 1% Triton X-100 (Nacalai Tesque), and a protease inhibitor cocktail (Nacalai Tesque). The lysate was centrifuged at 15 000 rpm for 5 min, and the supernatant was stored for subsequent analysis. Protein concentration was determined using DC protein assay reagents (Bio-Rad Laboratories, CA, USA) on the basis of the method of Lowry *et al.* [34]. Fifteen micrograms of protein was separated by 10% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, MA, USA). After blocking, the membrane was incubated with anti-inhibitor κB (IκB)-α (1:200; sc-371: Santa Cruz Biotechnology, CA, USA), anti-c-Jun N-terminal kinase (JNK) (1:1000; cat. no. 9258: Cell Signaling Technology, MA, USA), anti-pJNK (1:1000; cat. no. 9251: Cell Signaling Technology), or β-actin (1:1000; cat. no. 4967: Cell Signaling Technology) antibody, and then with a secondary antibody conjugated to horseradish peroxidase (anti-rabbit IgG: 1:2000; cat. no. W4011: Promega) for 1 h. The secondary antibody staining was visualized by chemiluminescence immunoassay using a chemiluminescent horseradish peroxidase substrate (Millipore). Immunoreactive protein bands were quantified using Image J software.

### 2.7 Statistical analysis

The data are presented as mean ± SE. Data were assessed by one-way ANOVA and Dunnet's multiple comparison test. Differences were considered significant at $p < 0.05$.

## 3 Results

### 3.1 Effects of diosgenin on inflammatory changes caused by coculture of adipocytes and macrophages

In this study, none of the tested concentration of diosgenin affected the viability of RAW 264 cells and 3T3-L1 cells after treating for 24 h (data not shown). The secretion of TNF-α (Fig. 2A), MCP-1 (Fig. 2B), and NO (Fig. 2C) by differentiated 3T3-L1 cells or RAW 264 cells was very low when they were cultured separately (TNF-α: 0.07 ± 0.01, 0.04 ± 0.02 ng/mL; MCP-1: 4.32 ± 0.23, 0.36 ± 0.04 ng/mL; NO: 1.19 ± 0.10, 1.96 ± 0.03 μM, respectively). However, the coculture of these cells in the contact system revealed a marked increase in all these inflammatory factors. Diosgenin treatment in this coculture decreased the secretion of these inflammatory factors in a dose-dependent manner (Fig. 2).

### 3.2 Effects of diosgenin on inflammation in macrophages induced by the adipocyte-derived CM

Diosgenin is reported to act as an anti-inflammatory in macrophages [22]. To investigate the function of diosgenin that suppresses the inflammatory changes in the interaction

**Table 1.** Sequences of the primer sets used for real-time PCR

| Primers | Forward | Reverse | GenBank accession number |
|---|---|---|---|
| mTNF-α | ACACTCAGATCATCTTCTCAAAATTCG | GTGTGGGTGAGGAGCACGTAGT | NM_013693 |
| mMCP-1 | ATGCAGGTCCCTGTCATGCTTC | GGCATCACAGTCCGAGTCACAC | NM_011333 |
| miNOS | CCAAGCCCTCACCTACTTCC | CTCTGAGGGCTGACACAAGG | BC062378 |
| m36B4 | TGTGTGTCTGCAGATCGGGTAC | CTTTGGCGGGATTAGTCGAAG | BC011291 |

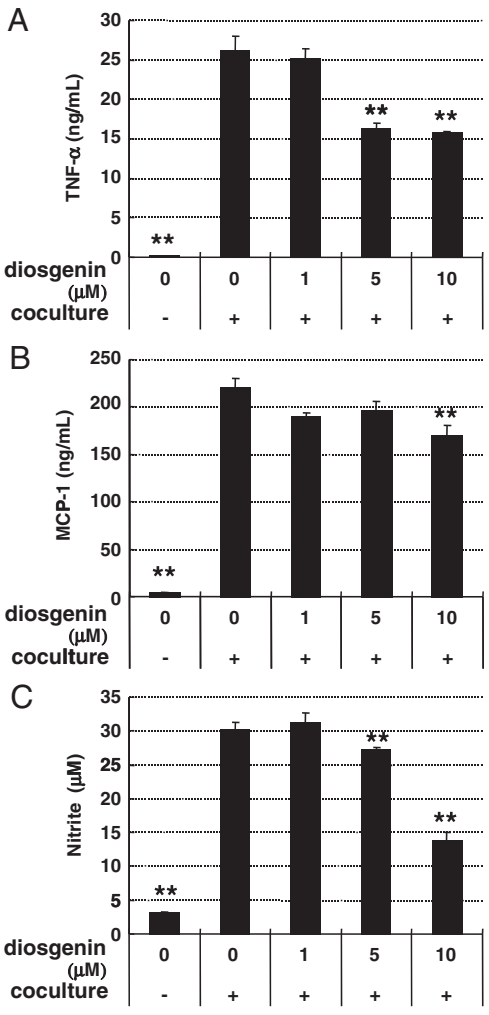



**Figure 2.** Effect of diosgenin on inflammatory changes induced by the coculture of 3T3-L1 adipocytes and RAW 264 macrophages. Differentiated 3T3-L1 adipocytes were cocultured with RAW 264 macrophages ($3 \times 10^5$ cells/well) for 24 h. TNF-α (A) and MCP-1 (B) levels in the coculture medium were measured by ELISA. The amount of secretion of NO (C) was also measured. As a control culture, we cultured 3T3-L1 cells and RAW264 cells separately, and measured the secretion levels in separate culture medium, taking the value for the control culture as the sum of the separate cultures. The control culture [diosgenin 0/coculture (−)] values are means ± SE of four replicants. $^{**}p < 0.01$ *versus* nontreated coculture.

of adipocytes and macrophages, we investigated the effect of diosgenin on the inflammation induced in macrophages by the CM derived from adipocytes. The CM derived from fully differentiated 3T3-L1 adipocytes significantly enhanced the production of TNF-α, MCP-1, and NO in RAW 264 macrophages (Fig. 3). However, diosgenin inhibited both the secretion (Figs. 3A–C) and the mRNA expression (Figs. 3D–F) of these inflammatory factors in a dose-dependent manner. These results suggest that diosgenin can suppress the inflammatory changes in macrophages in obese adipose tissues.

### 3.3 Effects of diosgenin on degradation of IκB-α and phosphorylation of JNK in macrophages

To clarify the mechanism by which diosgenin inhibits the CM-induced inflammation in macrophages, the degradation of IκB-α, which leads to the activation of NF-κB, and the phosphorylation of JNK were examined in RAW 264 cells treated with CM.

Although treatment with the CM from 3T3-L1 adipocytes for 30 min markedly promoted IκB-α degradation in RAW 264 macrophages, the degradation was inhibited by diosgenin in a dose-dependent manner (Fig. 4A). The CM-induced phosphorylation of JNK1/2 was also inhibited by diosgenin treatment in a dose-dependent manner (Fig. 4B).

## 4 Discussion

Diosgenin, a saponin aglycon based on a $C_{27}$ cholestane skeleton, has a large variety of biological functions including anti-inflammatory activity [22]. In this study, we investigated whether diosgenin could also act as an anti-inflammatory in the interaction of adipocytes and macrophages.

Although the coculture of fully differentiated 3T3-L1 adipocytes and RAW 264 macrophages markedly enhanced the production of inflammatory factors such as MCP-1, TNF-α, and NO, which was consistent with our previous report [29, 30], diosgenin significantly inhibited the coculture-induced inflammation. This result suggests that diosgenin can suppress the inflammatory changes in obese adipose tissues.

Numerous studies have implied that obesity is an inflammatory disease that can cause or worsen insulin resistance in adipose tissues, skeletal muscles, and the liver [35, 36]. Although the causes of this inflammation in obesity have not been entirely clarified, obese adipose tissues appear to play an important role in the relationship between obesity and chronic inflammation. Obese adipose tissues are characterized by the enhanced infiltration of macrophages [4, 5], and it is considered that a paracrine loop involving adipocyte-derived FFA and MCP-1 and macrophage-derived TNF-α establishes a vicious cycle that aggravates the inflammatory changes and insulin resistance in adipose tissues [11].

A previous report has demonstrated that saponin aglycons, including diosgenin, exhibit anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophages [22]. Therefore, we investigated the effect of diosgenin on the inflammation in macrophages induced by the CM derived from fully differentiated adipocytes. As is the case for the coculture of adipocytes and macrophages, the treatment of macrophages with the CM derived from adipocytes markedly enhanced the production of MCP-1, TNF-α, and NO. Diosgenin suppressed the production of these inflammatory factors in a dose-dependent manner. The CM derived from adipocytes has been reported to enhance the expression of TNF-α in macrophages, and this induction is considered to be caused

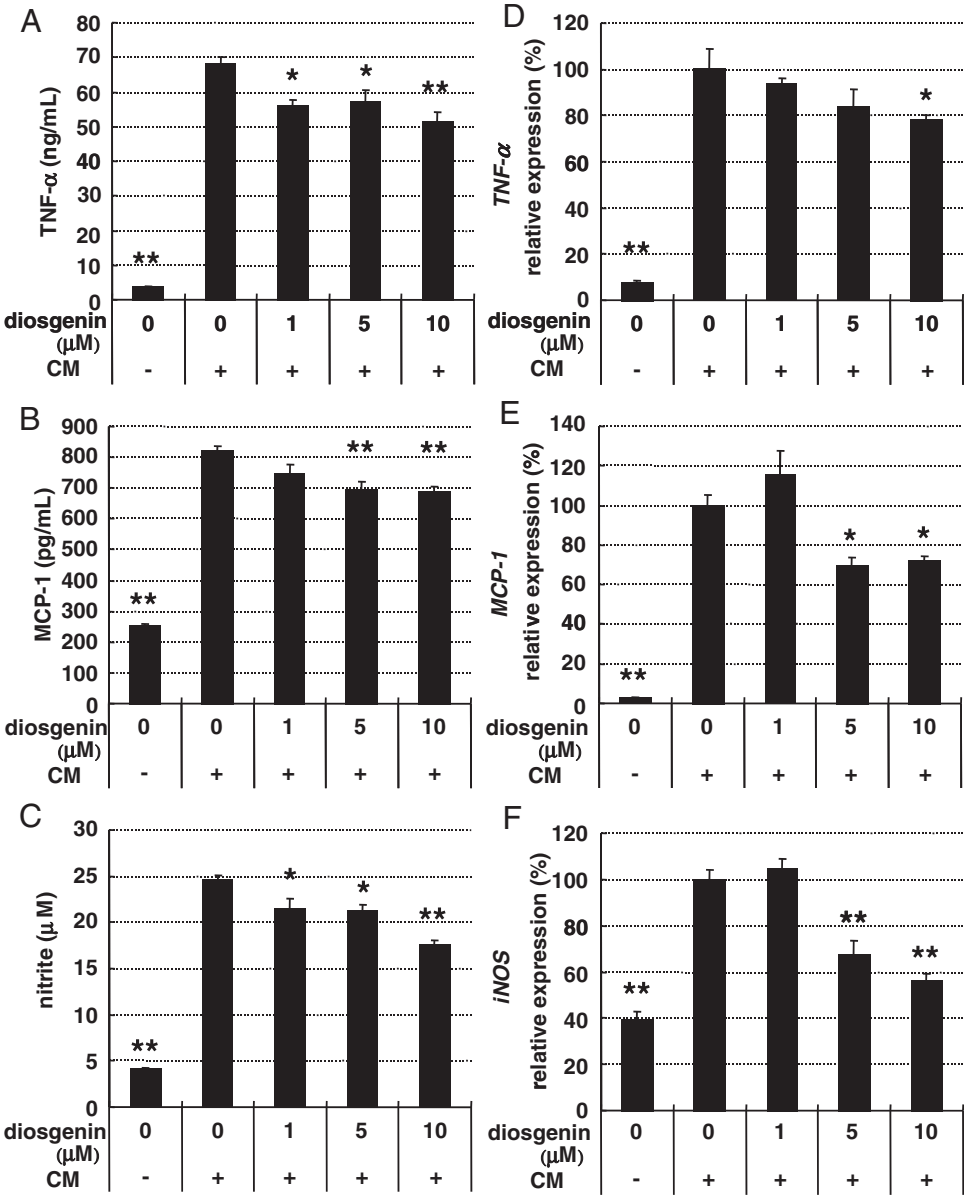



**Figure 3.** Effect of diosgenin on inflammatory changes in macrophages induced by the CM derived from adipocytes. RAW 264 macrophages ($5 \times 10^5$ cells/well) were treated with the CM derived from fully differentiated 3T3-L1 adipocytes and various doses of diosgenin for 24 h. TNF-α (A), MCP-1 (B), and NO (C) levels in the culture medium were measured as shown in Fig. 2 legend. The mRNA expression levels of TNF-α (D), MCP-1 (E), and inducible NO synthase (F) were measured by real-time PCR. Values are means ± SE of four replicants. $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* culture treated with the CM alone.

by FFA in the adipocyte-derived CM [11]. FFA released from adipocytes by the lipolysis of triglyceride exerts its proinflammatory effect on macrophages through the activation of TLR4, which is known as an essential cell surface receptor for the recognition of LPS in macrophages [37]. Downstream signals of TLR4 in LPS-stimulated macrophages have been well studied. It has been reported that the IκB/NF-κB pathway and three MAPK pathways, extracellular signal-regulated kinase, p38, and JNK, are involved in the TLR4 signals [6]. These signaling pathways activate a variety of transcription factors including NF-κB (p50/p65) [6] and activator protein-1 (AP-1) (c-Fos/c-jun) [38], which coordinately induce the transcription of many genes that encode inflammatory mediators. Suganami *et al.* [11, 39] reported that the FFA-induced activation of macrophages through TLR4 is also mediated by NF-κB, extracellular signal-regulated kinase, and JNK. Furthermore, the steroid saponin tomatidine, which is one of the nitrogen analoge of diosgenin, has been recently reported to act as an anti-inflammatory agent by inhibiting NF-κB and JNK signaling [22]. Consistent with these reports, our results showed that diosgenin inhibited the degradation of IκB-α, which is an inhibitory binding protein of NF-κB, as well as the phosphorylation of JNK in RAW 264 macrophages treated with the 3T3-L1-derived CM. These results suggest that diosgenin attenuates FFA-induced inflammation in macrophages *via* the suppression of both IκB/NF-κB and JNK/AP-1 signaling pathways.

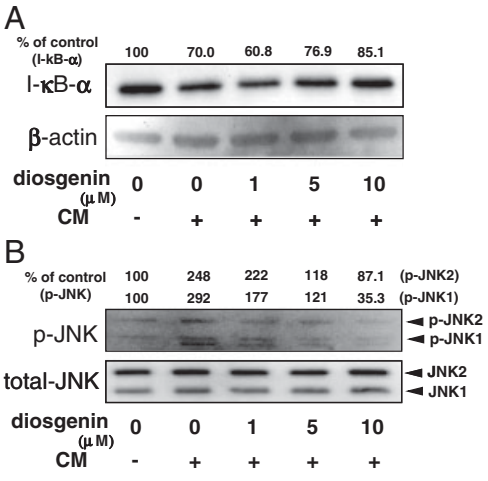



**Figure 4.** Effects of diosgenin on IκB-α degradation (A) and JNK phosphorylation (B) in RAW 264 macrophages stimulated with the CM derived from adipocytes. RAW 264 cells ($1 \times 10^6$ cells/well) were stimulated with the CM derived from fully differentiated 3T3-L1 adipocytes and various doses of diosgenin for 30 min (A) or 1 h (B). Total cell lysates were extracted from cultured RAW 264 cells, and IκB-α (37 kDa), β-actin (45 kDa), phosphorylated JNK (p-JNK1: 46 kDa, p-JNK2: 54 kDa), and total JNK (JNK1: 46 kDa, JNK2: 54 kDa) protein expressions were analyzed by Western blotting.

In obese adipose tissues, the increased production of TNF-α through the activation of the FFA/TLR4 signaling pathway in macrophages stimulates lipolysis in adipocytes to enhance the inflammatory changes in adipocytes and macrophages [11]. In this study, diosgenin also inhibited TNF-α-induced inflammation in 3T3-L1 adipocytes; however, it did not affect TNF-α-induced lipolysis in adipocytes (data not shown). It has been reported that the regulation of TNF-α-induced lipolysis is independent of the NF-κB pathway, whereas the TNF-α-induced inflammation in adipocytes is mediated by NF-κB and MAPK activation [39]. Therefore, diosgenin might also interfere with the NF-κB pathway in adipocytes and inhibit inflammation, but is not thought to interfere with lipolysis in adipocytes.

Diosgenin is structurally similar to glucocorticoids, which have also been shown to have anti-inflammatory properties [40]. Multiple mechanisms are involved in glucocorticoid-mediated anti-inflammatory activities; in addition to their direct effect on IκB/NF-κB activity [41, 42], glucocorticoids normally act by binding to their receptors in the cytoplasm and entering the nucleus, where they bind to DNA at glucocorticoid response elements, resulting in further interference with inflammatory transcription factors such as NF-κB and AP-1 [40, 43]. Thus, diosgenin might also interact with glucocorticoid response elements *via* glucocorticoid receptors in macrophages and exhibit anti-inflammatory properties in obese adipose tissue. Further studies are needed to clarify the glucocorticoid-like effect of diosgenin in macrophages.

The effect of dietary diosgenin on obesity-related chronic inflammation has not yet been reported; however, we have recently found that fenugreek, which contains a large amount of diosgenin, inhibited the infiltration of macrophages into adipose tissues in diabetic obese KKAy mice fed a high-fat diet (unpublished data), suggesting the possibility that diosgenin actually attenuates the inflammatory changes in obese adipose tissues *in vivo* and suppresses the migration of macrophages into adipose tissues.

Recently, inflammatory leukocytes, including T lymphocytes, were also reported to infiltrate obese adipose tissues, which may contribute to the local inflammatory cell activation [44]. Diosgenin has been reported to block the interaction of CXCR3 receptor expressed on the cell surface of T cells with its ligands produced by the inflammatory damage sites [26]. Therefore, diosgenin might also contribute to the attenuation of inflammatory changes in obese adipose tissues *via* suppressing T-cell recruitment to obese adipose tissues.

In summary, the results presented here showed that diosgenin suppresses the inflammatory changes in the interaction of adipocytes and macrophages by inhibiting the inflammatory signals in macrophages. Diosgenin may be valuable as a functional phytochemical for suppressing the vicious cycle of chronic inflammation in obese adipose tissues to improve obesity-related insulin resistance.

*This work was supported by the Research and Development Program for New Bio-industry Initiatives and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (15081205, 19380074, and 19780096). R. Y. was supported by a grant from the Korean Science and Engineering Foundation (KOSEF R01-2005-000-10408-0).*

*The authors have declared no conflict of interest.*

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