

# $\gamma$ -Oryzanol Reduces Adhesion Molecule Expression in Vascular Endothelial Cells via Suppression of Nuclear Factor- $\kappa$ B Activation

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**ABSTRACT:**  $\gamma$ -Oryzanol ( $\gamma$ -ORZ) is a mixture of phytosteryl ferulates purified from rice bran oil. In this study, we examined whether  $\gamma$ -ORZ represents a suppressive effect on the lipopolysaccharide (LPS)-induced adhesion molecule expression on vascular endothelium. Treatment with LPS elevated the mRNA expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin in bovine aortic endothelial cells (BAECs). Pretreatment with  $\gamma$ -ORZ dose-dependently decreased the LPS-mediated expression of these genes. Western blotting also revealed that pretreatment with  $\gamma$ -ORZ dose-dependently inhibited LPS-induced VCAM-1 expression in human umbilical vein endothelial cells. Consistently, pretreatment with  $\gamma$ -ORZ dose-dependently reduced LPS-induced U937 monocyte adhesion to BAECs. In immunofluorescence, LPS caused nuclear factor- $\kappa$ B (NF- $\kappa$ B) nuclear translocation in 40% of BAECs, which indicates NF- $\kappa$ B activation. Pretreatment with  $\gamma$ -ORZ, as well as its components (cycloartenyl ferulate, ferulic acid, or cycloartenol), dose-dependently inhibited LPS-mediated NF- $\kappa$ B activation. Collectively, our results suggested that  $\gamma$ -ORZ reduced LPS-mediated adhesion molecule expression through NF- $\kappa$ B inhibition in vascular endothelium.

**KEYWORDS:** Adhesion molecules, antioxidants, atherosclerosis,  $\gamma$ -oryzanol, nuclear factor- $\kappa$ B

## INTRODUCTION

Atherosclerosis is a progressive inflammatory disease of large arteries characterized by oxidized lipid accumulation on the vascular wall, neointima formation, fibrous cap formation, and inflammatory cell migration.<sup>1</sup> Impairment of vascular endothelial cells is involved in all stages of atherosclerosis.<sup>2</sup> Especially in the initial phase of atherosclerosis, injured endothelial cells increase the expression of adhesion molecules, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), which facilitates leukocyte adhesion to endothelium and migration to the subendothelial area.<sup>3</sup> Migrating leukocytes, especially monocytes secrete pro-inflammatory cytokines, scavenge oxidized low-density lipoprotein, and finally, disrupt. These phenomena result in injuring the vascular wall and augment inflammation.<sup>1,4,5</sup> Thus, reducing adhesion molecule expression and/or its signal blockade is assumed to be an attractive ways to prevent and treat atherosclerosis.

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is one of the major transcription factors that regulates pro-inflammatory gene expression. In inactivated cells, NF- $\kappa$ B is located in the cytoplasm. Inflammatory stimuli, such as inflammatory cytokines, oxidative stress, and lipopolysaccharide (LPS), cause translocation of NF- $\kappa$ B into the nucleus. The translocated NF- $\kappa$ B binds to specific sequences of DNA and modulates gene expressions.<sup>6,7</sup> Several studies showed that NF- $\kappa$ B regulates gene expression of adhesion molecules in vascular endothelium, including VCAM-1, ICAM-1, and E-selectin.<sup>7–10</sup> Thus, a suppressive agent against NF- $\kappa$ B has emerged as a promising candidate for an anti-arteriosclerotic drug for its potential anti-adhesion effect.

$\gamma$ -Oryzanol ( $\gamma$ -ORZ), purified from rice bran oil, contains several phytosteryl ferulates, including cycloartenyl ferulate (CAF) (Figure 1), 24-methylenecycloartenyl ferulate, and  $\beta$ -sitosteryl ferulate.<sup>11</sup>  $\gamma$ -ORZ and rice bran oil decrease serum total cholesterol by inhibiting lipid absorption from intestine in both human and animal models.<sup>12–14</sup> We previously reported that  $\gamma$ -ORZ and CAF have an anti-inflammatory effect in the dextran sulfate sodium colitis mice model and LPS-stimulated RAW 264.7 macrophages.<sup>15,16</sup> Furthermore, we showed that CAF, cycloartenol (CA), and ferulic acid (FA, *trans*-4-hydroxy-3-methoxycinnamic acid) (Figure 1), two hydrolytic products of CAF, inhibit NF- $\kappa$ B activation via its antioxidant activity in RAW 264.7 macrophages.<sup>17</sup> However, its effect on inflamed vascular endothelial cells is yet to be investigated. In this study, we investigated whether and how  $\gamma$ -ORZ represents suppressive effects on the LPS-induced adhesion molecule expression on vascular endothelial cells.

## MATERIALS AND METHODS

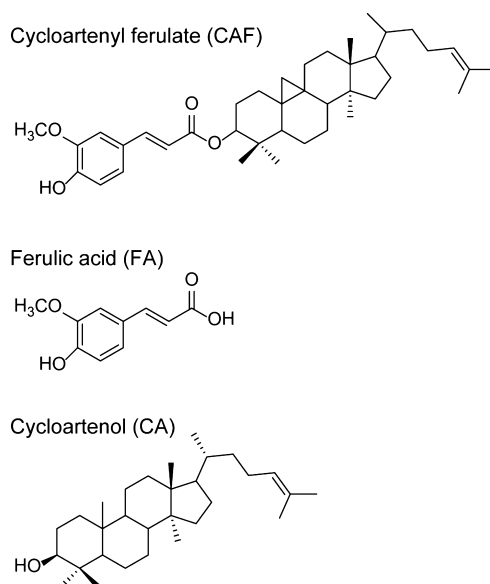
**Drugs and Reagents.** The drugs and reagents used in this experiment were described below.  $\gamma$ -ORZ was purified from Japanese rice, Koshi-hikari (*Oryza sativa*), as previously described.<sup>18</sup> Briefly, the fraction containing  $\gamma$ -ORZ was prepared from commercial rice bran oil through conventional silica gel column chromatography. Reverse-phase high-performance liquid chromatography (HPLC) revealed that

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**Figure 1.** Chemical structures of CAF, FA, and CA. Both FA and CA are hydrolytic products of CAF.

the  $\gamma$ -ORZ fraction was mainly composed of four major ferulates: CAF, 24-methylene cycloartenyl ferulate,  $\beta$ -sitosteryl ferulate, and campesterol ferulate. The average molecular weight of  $\gamma$ -ORZ was calculated from the molar ratio of these ferulates. CAF was purified from  $\gamma$ -ORZ by repeated recrystallization. CA was prepared by alkaline hydrolysis of CAF. FA was purchased from Wako Chemical (Japan). LPS from *Escherichia coli* O55:H5, pyrrolidine dithiocarbamate (PDTC), Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI), and mouse anti- $\beta$ -actin antibody were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). TRIzol reagent and ethidium bromide were purchased from Invitrogen (Carlsbad, CA). The 5 $\times$  RT buffer, 9-mer random primer, ReverTra Ace, and human placenta ribonuclease inhibitor were purchased from Toyobo Life Science (Japan). The 10 $\times$  *Ex taq* buffer, dNTP mixture, and *TaKaRa Ex taq* were purchased from Takara Bio, Inc. (Japan). Normal goat serum was purchased from Chemicon International (Temecula, CA). Rabbit polyclonal anti-NF- $\kappa$ B p65 and goat anti-VCAM-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 568-conjugated anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR). Donkey anti-goat DyLight 680 and goat anti-mouse IRDye 700DX antibodies were obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Guava Check Kit and Guava ViaCount Reagent were purchased from Millipore (Billerica, MA). Pefabloc SC and Complete Protease Inhibitor Cocktail were obtained from Roche Applied Science (Indianapolis, IN). EGM-2 bullet kit and EBM-2 were obtained from Lonza (Switzerland).

**Cell Culture.** Bovine aortic endothelial cells (BAECs) were isolated from bovine thoracic aortas purchased from a slaughterhouse and cultured in DMEM with 10% FBS. BAECs between passages 8 and 12 were used. BAECs were serum-starved in DMEM with 1% FBS for 24 h before the experiments. Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza and cultured in EGM-2. HUVECs between passages 6 and 9 were used. HUVECs were serum-starved in EBM with 2% FBS overnight before the experiments. U937 monocytes (human leukemic lymphoma cell line) were obtained from RIKEN Cell Bank (Japan) and cultured in RPMI 1640 medium with 10% FBS.

**Total RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR).** Total RNA extraction, the first strand cDNA synthesis, and PCR were performed as described previously.<sup>19</sup> Briefly, PCR was performed using 0.0125 unit/ $\mu$ L Taq DNA poly-

merase and 1  $\mu$ M primers. A total of 30 cycles of amplification was performed, each consisted of 98  $^{\circ}$ C for 10 s, 57  $^{\circ}$ C for 30 s, and 72  $^{\circ}$ C for 60 s using a thermal cycler (Takara PCR Thermal Cycler MP, Bio, Inc., Japan). The primers were designed as follows: VCAM-1: sense, 5'-GAACCGACAGCTCCTTTCTG-3'; antisense, 5'-TCCCTGACATCACAGGTCAA-3'; ICAM-1: sense, 5'-GCAACTTCTCCTGCTCTGCT-3'; antisense, 5'-TTCCGGCTACAGTTCAGCTT-3'; E-selectin: sense, 5'-GCACAGAAACATCCTGAGCA-3'; antisense, 5'-ACATTGCATTTTGGGAGAGG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): sense, 5'-CAGGGCTGCTTTTAATTCTG-3'; antisense, 5'-AGCACCAGCATCACCAACT-3'. The products were electrophoresed in 2.0% agarose gels, and the densitometric intensity of the bands (VCAM-1, 221 bp; ICAM-1, 201 bp; E-selectin, 169 bp; and GAPDH, 220 bp) was quantified using ImageJ. The results were expressed as the ratios of optical density of each product to that of GAPDH.

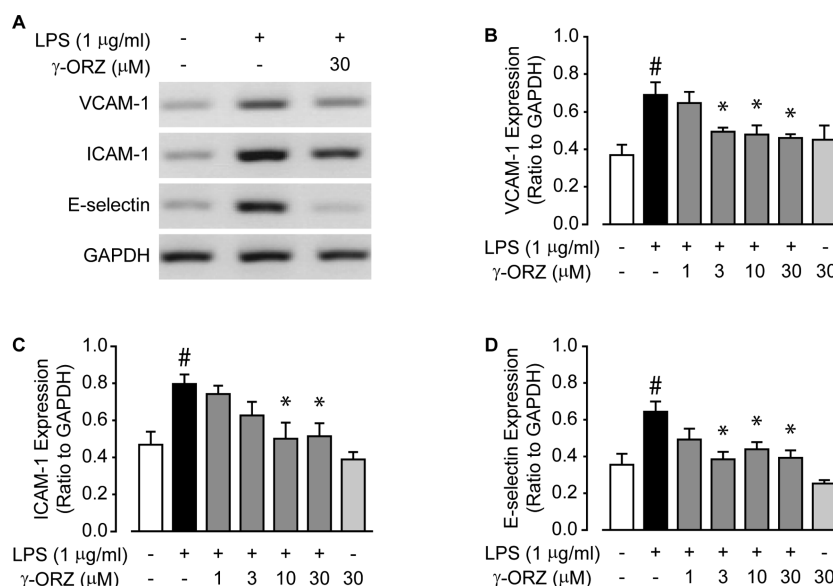
**Total Protein Extraction and Western Blotting.** HUVECs were lysed in ice-cold lysis buffer containing 1% Triton X-100, 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerol phosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu$ g/mL Pefabloc SC, and 1  $\mu$ g/mL Complete (protease inhibitor cocktail) and centrifuged at 15 000 rpm for 15 min at 4  $^{\circ}$ C. Proteins (20  $\mu$ g) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5%) and transferred to a nitrocellulose membrane. After the membranes were blocked with blocking buffer containing 3% BSA, they were probed with anti-VCAM-1 antibody (1:300) or anti- $\beta$ -actin antibody (1:4000) overnight at 4  $^{\circ}$ C. For the secondary antibody reaction, membranes were incubated with corresponding secondary antibodies (both 1:10 000) for 1 h at room temperature and bands were detected and quantified with the Odyssey system (LI-COR Biosciences, Lincoln, NE). The results were expressed as a ratio of VCAM-1/ $\beta$ -actin.

**Adhesion Assays.** Confluent BAECs were stimulated with LPS (1  $\mu$ g/mL, 6 h) after serum starvation. After washing twice with serum-free DMEM,  $2.5 \times 10^5$  cells/mL of U937 were added onto BAECs and incubated with oval shaking (30 rpm, 30 min, 37  $^{\circ}$ C). After the incubation, non-adherent U937 monocytes were washed away and then the cells were fixed in 4% paraformaldehyde (PFA) for 5 min. The number of U937 adhered to BAECs was randomly counted under ECLIPSE TS100-F microscope (Nikon, Japan). The results were expressed as the ratios of the number of U937 adhered to each sample to that of nontreated BAECs.

**Immunofluorescence.** BAECs were plated onto sterilized coverslips in 12-well plates and used after serum starvation. After stimulation with LPS (1  $\mu$ g/mL, 2 h), the cells were fixed with 4% PFA for 5 min and permeabilized with 0.2% Triton X-100 for 5 min. After washing twice, the cells were incubated with blocking buffer containing 1% BSA and 3% normal goat serum for 1 h at room temperature. Then, they were incubated with primary antibody (1:250 dilution) overnight at 4  $^{\circ}$ C and then with secondary antibody (1:500 dilution) for 1 h at room temperature. Nuclei were labeled with DAPI (1  $\mu$ g/mL). The images were captured using ECLIPSE E800 fluorescence microscope (Nikon, Japan), and the number of BAECs showing NF- $\kappa$ B nuclear translocation was randomly counted.

**Viability Assays.** Confluent BAECs were incubated with  $\gamma$ -ORZ, CAF, FA, and CA (30  $\mu$ M each) for 24 h after serum starvation. The percentages of viable cells were quantified with the Guava PCA (Millipore, Billerica, MA) according to the protocol of the manufacturer. This assay distinguishes viable and nonviable cells based on differential permeabilities of two DNA-binding dyes in the Guava ViaCount Reagent.

**Statistical Analysis.** The data are expressed as the mean  $\pm$  standard error of the mean (SEM). Statistical evaluation of the data was carried out by unpaired Student's *t* test for comparison between two groups and one-way analysis of variation followed by Dunnett's test for the comparisons among more than two groups. A value of *p* < 0.05 was taken as statistically significant.



**Figure 2.**  $\gamma$ -ORZ reduces LPS-induced adhesion molecule expression in BAECs. BAECs were pre-incubated with  $\gamma$ -ORZ (1–30  $\mu$ M, 16 h) and then stimulated with LPS (1  $\mu$ g/mL, 3 h). After LPS stimulation, total RNA was extracted and then RT-PCR was performed. (A) Typical traces of agarose gel electrophoresis of RT-PCR products. (B–D) Quantification of the (B) VCAM-1, (C) ICAM-1, and (D) E-selectin mRNA expression level. Results are the mean  $\pm$  SEM ( $n = 4$ –7). (#) Significantly different from the results in nontreated cells at  $p < 0.05$ . (\*) Significantly different from the results in LPS-treated cells at  $p < 0.05$ .

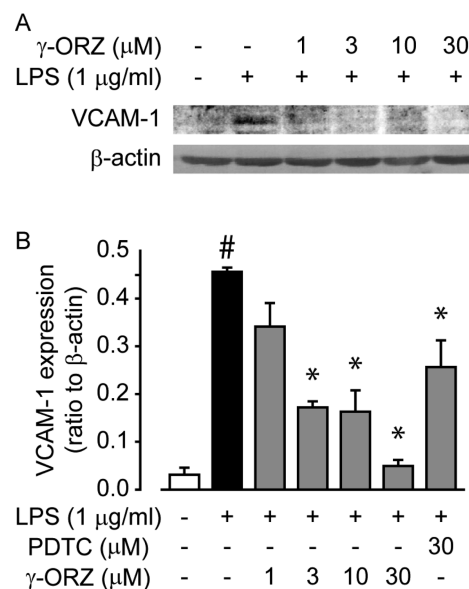
## RESULTS

**$\gamma$ -ORZ Inhibits mRNA Expression of Adhesion Molecules in BAECs.** We investigated whether  $\gamma$ -ORZ has an inhibitory effect on the mRNA expression of adhesion molecules (VCAM-1, ICAM-1, and E-selectin) in vascular endothelial cells. LPS (1  $\mu$ g/mL, 3 h) stimulation upregulated the expression of these adhesion molecules (intensity ratio to GAPDH: VCAM-1,  $0.37 \pm 0.06$ ; ICAM-1,  $0.47 \pm 0.07$ ; and E-selectin,  $0.35 \pm 0.06$  for the nontreated control and VCAM-1,  $0.69 \pm 0.07$ ; ICAM-1,  $0.80 \pm 0.05$ ; and E-selectin,  $0.64 \pm 0.06$  for the LPS-stimulated group;  $n = 8$ –9;  $p < 0.05$ ) (Figure 2).

Pretreatment with  $\gamma$ -ORZ (3  $\mu$ M, 16 h) significantly inhibited VCAM-1 and E-selectin but not ICAM-1 mRNA expression. Pretreatment with the higher concentration of  $\gamma$ -ORZ (10–30  $\mu$ M, 16 h) significantly reduced all of these adhesion molecule mRNA expressions (intensity ratio to GAPDH:  $0.46 \pm 0.02$  for VCAM-1,  $0.51 \pm 0.07$  for ICAM-1, and  $0.39 \pm 0.04$  for E-selectin; under 30  $\mu$ M  $\gamma$ -ORZ pretreatment;  $n = 6$ –7 each;  $p < 0.05$ ) (Figure 2).

**$\gamma$ -ORZ Reduces Adhesion Molecule Protein Expression in HUVECs.** To confirm that  $\gamma$ -ORZ reduces the expression of adhesion molecules, we performed western blot analysis in HUVECs. LPS (1  $\mu$ g/mL, 6 h) stimulation increased VCAM-1 expression (ratio to  $\beta$ -actin:  $0.0309 \pm 0.0149$  for nontreated control and  $0.456 \pm 0.09$  for the LPS-stimulated group;  $n = 4$  each;  $p < 0.05$ ) (Figure 3). As expected from the result of RT-PCR (Figure 2), pretreatment of  $\gamma$ -ORZ (3–30  $\mu$ M, 16 h) significantly reduced LPS-induced VCAM-1 expression in HUVECs (ratio to  $\beta$ -actin:  $0.0493 \pm 0.0128$ ;  $n = 4$ ;  $p < 0.05$ ) (Figure 3).

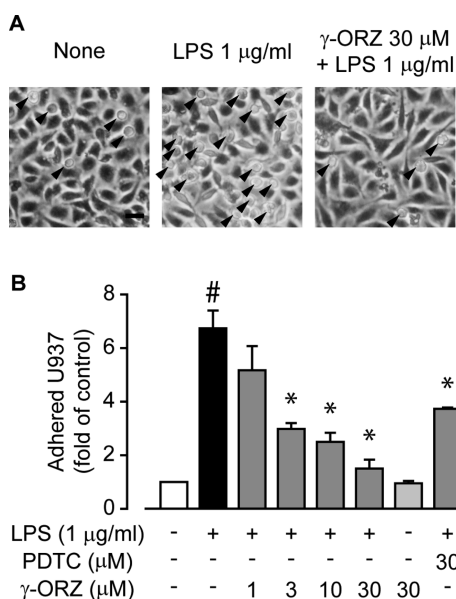
**$\gamma$ -ORZ Reduces U937 Monocyte Adhesion to BAECs.** To investigate the effect of  $\gamma$ -ORZ on the leukocyte adhesion to vascular endothelium, we performed adhesion assays using U937 monocytes. LPS (1  $\mu$ g/mL, 6 h) stimulation significantly increased U937 adhesion to BAECs (fold of nontreated control:  $7.00 \pm 0.60$ ;  $n = 6$ ;  $p < 0.05$ ) (Figure 4).



**Figure 3.**  $\gamma$ -ORZ reduces LPS-induced VCAM-1 protein expression in HUVECs. HUVECs were pre-incubated with  $\gamma$ -ORZ (1–30  $\mu$ M, 16 h) or PDTc (30  $\mu$ M, 1 h) and then stimulated with LPS (1  $\mu$ g/mL, 6 h). (A) Typical picture of electrophoresis. (B) Quantification of the VCAM-1 protein expression level. Results are the mean  $\pm$  SEM ( $n = 4$ ). (#) Significantly different from the results in nontreated cells at  $p < 0.05$ . (\*) Significantly different from the results in LPS-treated cells at  $p < 0.05$ .

Consistent with the results of adhesion molecule expression (Figure 2 and 3), pretreatment of BAECs with  $\gamma$ -ORZ (3–30  $\mu$ M, 16 h) significantly reduced LPS-induced monocyte adhesion to BAECs (fold of nontreated control:  $1.70 \pm 0.32$  for 30  $\mu$ M;  $n = 5$ ;  $p < 0.05$ ) (Figure 4). Pretreatment with PDTc (30  $\mu$ M, 1 h), a synthetic antioxidant known as a NF- $\kappa$ B inhibitor, also significantly decreased monocyte adhesion to BAECs (fold of nontreated control:  $3.73 \pm 0.05$ ;  $n = 4$ ;  $p < 0.05$ ) (Figure 4).





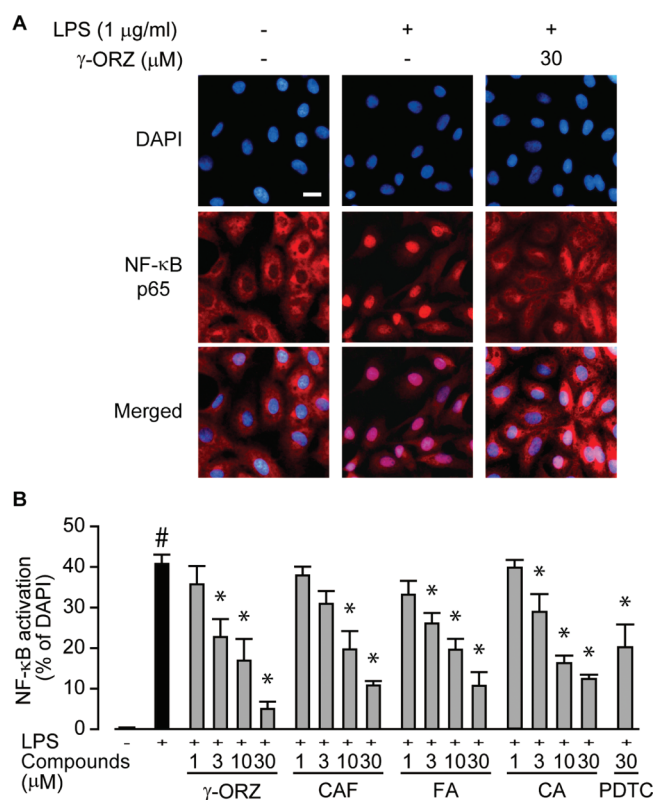
**Figure 4.** γ-ORZ reduces U937 monocyte adhesion to BAECs. BAECs were pre-incubated with γ-ORZ (1–30 µM, 16 h) or PDTC (30 µM, 1 h) and then stimulated with LPS (1 µg/mL, 6 h). A total of  $2.5 \times 10^5$  cells/mL of U937 were added onto BAEC monolayers. After the incubation, the number of U937 monocytes adhered to BAEC monolayers was counted. (A) Typical photographs of U937 monocytes adhered to BAEC monolayers. Arrowheads show adhered U937 monocytes. Scale bar = 50 µm. (B) Quantification of the U937 adhesion. The number of U937 monocytes adhered to nontreated BAECs was taken as 1. Results are the mean ± SEM ( $n = 4-7$ ). (#) Significantly different from the results in nontreated cells at  $p < 0.05$ . (\*) Significantly different from the results in LPS-treated cells at  $p < 0.05$ .

γ-ORZ showed about 2-fold inhibitory efficacy of PDTC at the same concentration (30 µM).

**γ-ORZ Inhibits NF-κB Activation in BAECs.** There is the possibility that γ-ORZ attenuates the LPS-induced inflammatory responses in endothelial cells through NF-κB inactivation. Therefore, we next investigated the effect of γ-ORZ on NF-κB activation in BAECs, observing NF-κB p65 translocation into nuclei. LPS (1 µg/mL, 2 h) stimulation induced NF-κB p65 nuclear translocation in BAECs (percentages of DAPI: nontreated control,  $0.33 \pm 0.39$ ; LPS-stimulated group,  $39.8 \pm 2.5\%$ ;  $n = 19-20$ ;  $p < 0.05$ ) (Figure 5).

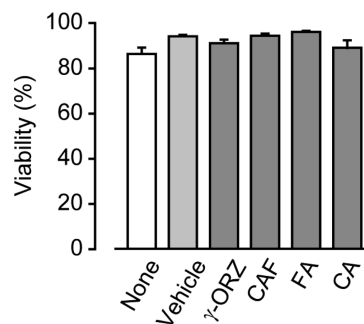
Pretreatment with γ-ORZ (3–30 µM, 16 h) remarkably inhibited LPS-induced NF-κB p65 translocation (percentage of DAPI:  $5.0 \pm 1.8\%$  for 30 µM;  $n = 7$ ;  $p < 0.05$ ) (Figure 5). We also investigated whether CAF, FA, and CA have a NF-κB inhibitory effect. Similar to γ-ORZ, all of these chemicals inhibited NF-κB activation in a dose-dependent manner (Figure 5). Pretreatment with PDTC (30 µM, 1 h) also inhibited LPS-induced NF-κB activation in BAECs (percentage of DAPI:  $20.2 \pm 5.6\%$ ;  $n = 4$ ;  $p < 0.05$ ) (Figure 5). Consistent with the results of RT-PCR (Figure 2), western blotting (Figure 3) and adhesion assays (Figure 4), γ-ORZ showed stronger NF-κB inhibition than PDTC at the same concentration (30 µM).

**γ-ORZ Does Not Affect the Viability of BAECs.** To confirm whether γ-ORZ has some cytotoxic effect on BAECs, we performed a viability assay using the Guava PCA system. Chronic treatment of BAECs with γ-ORZ (30 µM, 24 h) did not influence the percentage of viable cells compared to vehicle-treated cells (percentages of viable cells:  $86.4 \pm 2.8\%$  for nontreated cells,  $94.2 \pm 0.7\%$  for vehicle-treated cells,



**Figure 5.** γ-ORZ and its components inhibit NF-κB nuclear translocation in BAECs. BAECs were pre-incubated with γ-ORZ, CAF, FA, CA (1–30 µM, 16 h), or PDTC (30 µM, 1 h) and then stimulated with LPS (1 µg/mL, 2 h). The p65 subunit of NF-κB was indirectly stained with Alexa-Fluor-568-labeled antibody (red), and nuclei were stained with DAPI (blue). (A) Typical photographs under the fluorescence microscope. Scale bar = 10 µm. (B) Graphical representation of percentage of NF-κB activated cells per field. Results are the mean ± SEM ( $n = 4-17$ ). (#) Significantly different from the results in nontreated cells at  $p < 0.05$ . (\*) Significantly different from the results in LPS-treated cells at  $p < 0.05$ .

and  $91.2 \pm 1.6\%$  for γ-ORZ-treated cells;  $n = 4-8$  each;  $p > 0.05$ ) (Figure 6). Similar to γ-ORZ, treatment with CAF, FA,



**Figure 6.** γ-ORZ and its components do not affect the viability of BAECs. Confluent BAECs were incubated with γ-ORZ, CAF, FA, CA (30 µM each), or vehicle for 24 h. The percentages of viable cells were measured using the Guava PCA system. Results are the mean ± SEM ( $n = 4-8$ ). No significant difference was observed.

and CA (30 µM, 24 h) showed no toxic effect on BAECs (percentages of viable cells:  $94.5 \pm 1.0\%$  for CAF,  $96.2 \pm 0.6\%$  for FA, and  $89.2 \pm 3.4\%$  for CA;  $n = 4-8$  each;  $p > 0.05$ ) (Figure 6).

## DISCUSSION

In the present study, we demonstrated that  $\gamma$ -ORZ reduces elevated expressions of adhesion molecules and monocyte adhesion to vascular endothelial cells. In detail, these beneficial effects of  $\gamma$ -ORZ are mainly due to NF- $\kappa$ B inhibition by its components.

$\gamma$ -ORZ is a mixture of phytosteryl ferulates purified from rice bran oil.<sup>11</sup> CAF is a major component of  $\gamma$ -ORZ.<sup>20</sup> In the present study, we found that all of the  $\gamma$ -ORZ-associated chemicals dose-dependently inhibited NF- $\kappa$ B activation in endothelial cells (Figure 5). These observations are consistent with our previous report showing that these chemicals reduced NF- $\kappa$ B activation in RAW 264.7 macrophages.<sup>17</sup>

Several studies demonstrated that LPS-induced inflammatory responses, including pro-inflammatory cytokine secretion and adhesion molecule expression, are mediated by intracellular reactive oxygen species (ROS)<sup>1</sup> production and subsequent activation of NF- $\kappa$ B.<sup>21,22</sup> We previously reported that all of the  $\gamma$ -ORZ components, CAF, FA, and CA, had an intracellular antioxidant effect in the NIH 3T3 fibroblasts.<sup>17</sup> Thus, the  $\gamma$ -ORZ-mediated anti-inflammatory effects accompanied with NF- $\kappa$ B inhibition in BAECs are likely to be due to the antioxidant potency of its components.

There are several types of NF- $\kappa$ B inhibitors, including PDTC and BAY11-7082. PDTC inhibits NF- $\kappa$ B activity via its antioxidant effect, while BAY11-7082 directly inhibits I $\kappa$ B- $\alpha$  phosphorylation. We previously reported that PDTC (30  $\mu$ M) or BAY11-7082 (10  $\mu$ M) significantly reduced LPS (3  $\mu$ g/mL)-induced adhesion molecule mRNA expression (percentages of decrease: 59.3% for VCAM-1, 20.3% for ICAM-1, and 78.3% for E-selectin under 30  $\mu$ M PDTC pretreatment and 77.8% for VCAM-1, 55.1% for ICAM-1, and 78.3% for E-selectin under 10  $\mu$ M BAY11-7082 pretreatment) (Figure 2).<sup>19</sup> In comparison to these established NF- $\kappa$ B inhibitors,  $\gamma$ -ORZ pretreatment generally represented stronger suppression on LPS-induced mRNA expression of adhesion molecules (percentages of decrease: 71.9% for VCAM-1, 87.9% for ICAM-1, and 86.2% for E-selectin under 30  $\mu$ M  $\gamma$ -ORZ pretreatment). Consistently, the suppressive effects of  $\gamma$ -ORZ on monocyte adhesion and NF- $\kappa$ B activation were stronger than PDTC (30  $\mu$ M, 1 h), even at lower concentrations (3–30  $\mu$ M) (Figures 4 and 5).

$\gamma$ -ORZ is considered to be partially hydrolyzed by cholesterol esterases and absorbed in the gastrointestinal system.<sup>23</sup> A recent study revealed that the plasma concentration of  $\gamma$ -ORZ increases up to 2  $\mu$ g/mL (3.3  $\mu$ M) at 4 h after oral administration of 20 mg/kg of  $\gamma$ -ORZ in rats.<sup>24</sup> Given that 3  $\mu$ M  $\gamma$ -ORZ represented anti-inflammatory effects in the present experiments (Figures 2–5),  $\gamma$ -ORZ might be beneficial in *in vivo* situations.

A previous report showed that  $\gamma$ -ORZ represents very low toxicity in experimental animals.<sup>25</sup> In an acute oral toxicity test, LD<sub>50</sub> values were all >4000 mg/kg in mice, rats, dogs, and rabbits. It also exhibited no chronic oral toxicity, reproductive toxicity, genotoxicity, and carcinogenicity.<sup>25</sup> In this experiment, we demonstrated that  $\gamma$ -ORZ and its related chemicals do not represent any evident cytotoxicity on BAECs at the *in vitro* level (Figure 6).

In conclusion, we showed that  $\gamma$ -ORZ reduces LPS-induced adhesion molecule mRNA expression in vascular endothelial cells and monocyte adhesion to them. It was also demonstrated that  $\gamma$ -ORZ is a very attractive NF- $\kappa$ B inhibitor, possessing strong potency and high safety. Inhibitory effects of  $\gamma$ -ORZ on

leukocyte adhesion to endothelial cells might be beneficial to reduce the risk of atherogenesis.

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

BAECs; bovine aortic endothelial cells; CA; cycloartenol; CAF; cycloartenyl ferulate; DAPI; 4',6'-diamidino-2-phenylindole; FA; ferulic acid; FBS; fetal bovine serum; GAPDH; glyceraldehyde 3-phosphate dehydrogenase; HUVECs; human umbilical vein endothelial cells; ICAM-1; intercellular cell adhesion molecule-1; LPS; lipopolysaccharide; NF- $\kappa$ B; nuclear factor- $\kappa$ B; PDTC; pyrrolidine dithiocarbamate; PFA; para-formaldehyde; ROS; reactive oxygen species; RPMI; Roswell Park Memorial Institute; VCAM-1; vascular cell adhesion molecule-1;  $\gamma$ -ORZ;  $\gamma$ -oryzanol

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