Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 490–497
Journal of Nutritional Biochemistry

Inhibition of macrophage adhesion activity by 9*trans*,11*trans*-conjugated linoleic acid

Yunkyoung Lee[a], John P. Vanden Heuvel[a,b,c,*]

[a] Integrative Biosciences, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802, USA
[b] Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, PA 16802, USA
[c] Center of Excellence in Nutrigenomics, Pennsylvania State University, University Park, PA 16802, USA

Received 15 October 2008; received in revised form 9 January 2009; accepted 11 February 2009

Abstract

Conjugated linoleic acids (CLAs) have anti-atherogenic effects in both in vitro and animal models. Most studies on CLAs were performed with either a CLA mixture or purified 9*cis* (*Z*),11*trans* (*E*)-CLA or 10*E*,12*Z*-CLA isomers. However, the 9*E*,11*E* isomer has superior anti-carcinogenic and anti-inflammatory effects compared with the more abundant CLAs. The 9*E*,11*E*-CLA isomer specifically increases interleukin-1 receptor antagonist (IL-1Ra), an important anti-inflammatory mediator that is associated with decreased risk of coronary heart disease. The purpose of this present study was to determine if 9*E*,11*E*-CLA affects markers of atherogenesis via regulation of IL-1Ra. In human umbilical vein endothelial cells (HUVECs), 9*E*,11*E*-CLA decreased such atherogenesis-related genes as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, E-selectin, P-selectin and C–C motif chemokine receptor-2. Treatment of RAW 264.7 cells with 9*E*,11*E*-CLA decreased their adhesion to HUVECs. This effect was reversed by inhibiting the phosphoinositide 3-kinase or mouse target of rapamycin pathways. IL-1Ra-deficient RAW 264.7 cells (siIL-1Ra RAW) bind more efficiently to HUVECs compared with the control stable cells (si-control RAW). In addition, HUVECs treated with siIL-1Ra RAW-conditioned media induce significantly higher levels of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, monocyte chemoattractant protein-1 and E-selectin than HUVECs treated with si-control RAW-conditioned media. Taken together, the data show that 9*E*,11*E*-CLA decreases the atherogenesis-related genes in HUVECs and alters adhesion of macrophages. In addition, the induction of IL-1Ra by 9*E*,11*E*-CLA is partially responsible for the anti-atherogenic properties of this particular CLA isomer.

Keywords: 9*E*,11*E*-CLA; Adhesion molecules; Macrophages; Endothelial cells

1. Introduction

Atherosclerosis is the primary cause of heart disease and stroke, collectively accounting for approximately 50% of all deaths in the United States [1]. Epidemiological studies have uncovered a number of risk factors for atherosclerosis, including high ratios of LDL/VLDL (low-density lipoprotein to very-low-density lipoprotein), low levels of HDL (high-density lipoprotein), high levels of homocysteine, systemic inflammation, metabolic syndrome, obesity and diabetes, high-fat diet, smoking and lack of exercise [1]. In spite of the importance of hypercholesterolemia in atherosclerosis and cardiovascular diseases, lipid-lowering drugs (statins) decreased cardiovascular incidence by only 30%, suggesting that other factors contribute to the development of atherosclerosis [2]. In 1999, atherosclerosis was first defined as an inflammatory disease [3]. Inflammation-related factors, such as C-reactive protein and the white blood cell number, are recognized as new biomarkers for atherosclerosis, which strongly support the definition of atherosclerosis. Recruitment of leukocytes to endothelial cells (ECs) is an important initial step for atherosclerosis. Functional changes on ECs are the initial event in the pathogenesis of atherosclerosis. The tethering and rolling of leukocytes to ECs are precisely controlled via a regulation of various adhesion molecules in ECs [3,4]. Monocytes attached to ECs migrate into the subendothelial space and consequently differentiate into macrophages. Macrophages uptake oxLDL (oxidized low-density lipoprotein) via scavenger receptors that lead to foam cell formation. Smooth muscle cells also migrate from the medial portion of the arterial wall and form a fibrous plaque via secreting extracellular matrix proteins, eventually leading to plaque rupture [3,4].

Conjugated linoleic acid (CLA) is a mixture of positional and geometrical isomers of LA (C18:2, n-6) that are generated by biohydrogenation and oxidation of LA [5,6]. The main food sources of CLA in the Western diet are meat and dairy products. During the last two decades, CLA has been intensively studied due to its various health-beneficial effects (reviewed by Wahle *et al.* [7]). Due to a predominance of 9*cis* (*Z*),11*trans* (*E*)-CLA and 10*E*,12*Z*-CLA in the diet and in dietary supplements, most studies were performed using these two isomers or a mixture of CLAs. CLA reduces the development of atherosclerosis by inhibiting lesion development and altering lipid

* Corresponding author. Department of Veterinary and Biomedical Sciences, Penn State University, University Park, PA 16802, USA. Tel.: +1 814 863 8532; fax: +1 814 863 1696.
E-mail address: jpv2@psu.edu (J.P. Vanden Heuvel).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2009.02.005

profile in animal models [8,9]. Possible targets for CLA activity include nuclear factor κB, peroxisome proliferator-activated receptors, sterol regulatory element-binding proteins and stearoyl-CoA desaturase [10,11]. In contrast, CLA increased fatty streak development in the aorta in a murine model of atherosclerosis [12]. 9*Z*,11*E*-CLA reduced intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) levels in human gastric carcinoma cells, resulting in decreased adhesion of the carcinoma cells to laminin [13]. On the other hand, Nugent et al. reported no significant change on prostaglandin E_2 and ICAM-1 in healthy humans on CLA mix (3 g/day) diet for 8 weeks [14]. Schleser et al. also showed no effect of 9*Z*,11*E*-CLA and 10*E*,12*Z*-CLA on adhesion molecules in human aortic ECs [14,15]. The discrepant results may be due to the isomer specificity of CLA and cell and species differences in response.

We previously showed that 9*E*,11*E*-CLA has unique anti-inflammatory properties compared with other CLA isomers (9*Z*,11*E*-CLA, 9*Z*,11*Z*-CLA, 10*E*,12*Z*-CLA or 11*Z*,13*E*-CLA) in its ability to induce interleukin-1 receptor antagonist (IL-1Ra) in a mouse macrophage cell line, RAW 264.7 [16]. IL-1Ra is an endogenous antagonist to IL-1 signaling that plays an important role in coronary artery disease [17,18]. In fact, an IL-1Ra polymorphism is closely associated with the risk of coronary artery disease [19]. We hypothesized that 9*E*,11*E*-CLA can affect atherogenic markers in part due to its unique ability to increase production of IL-1Ra. In this present study, we demonstrated effects of 9*E*,11*E*-CLA on atherogenesis-related genes in human umbilical vein endothelial cells (HUVECs) and decreased adhesion between macrophages and ECs.

2. Materials and methods

2.1. Materials

The CLA isomer 9*E*,11*E*-CLA (>98% purity) was purchased from Matreya (Pleasant Gap, PA, USA). Lipopolysaccharide (LPS), bovine serum albumin (BSA) and SIGMA FAST™ OPD were purchased from Sigma (Saint Louis, MO, USA). All primers for real-time PCR were designed by using Primer Express (Applied Biosystems, Foster City, CA, USA) and were purchased from either Operon Biotechnologies (Huntsville, AL, USA) or the Nucleic Acid Facility at the Pennsylvania State University (University Park, PA, USA). Real-time PCR reagent, SybrGreen and High-Capacity cDNA Archive Kit were purchased from Applied Biosystems. BCECF-AM [2,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester] and tumor necrosis factor-α (TNF-α) were purchased from Invitrogen (Carlsbad, CA, USA). LY294002 [phosphoinositide 3-kinase (PI3K) inhibitor] and rapamycin [mouse target of rapamycin (mTOR) inhibitor] were purchased from Calbiochem (La Jolla, CA, USA). Monoclonal anti-human VCAM-1 (CD106: VCAM-1) was purchased from R&D Systems (Minneapolis, MN, USA). All cell culture media components and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). RAW 264.7 cells were purchased from ATCC (Manassas, VA, USA), and HUVECs and EGM-2 (EC growth media-2) were purchased from Lonza (Allendale, NJ, USA).

2.2. Cell culture

RAW 264.7 (RAW) cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 8% heat-inactivated FBS, 5 mg/ml of ciprofloxacin, 2 mM L-glutamine, 100 mM sodium pyruvate and 100 μM non-essential amino acids [20]. Starve media were the RAW growth media with 1% heat-inactivated FBS. Stable cell lines were previously generated in RAW 264.7 cells (siIL-1Ra and its control cell line) and were cultured in RAW growth media with 0.3 mg/ml of Geneticin. Conditioned media collected from RAW cells were treated with BSA or 9*E*,11*E*-CLA (100 μM) with or without LPS (100 ng/ml) for 24 h. The media were centrifuged for 5 min at 5000 rpm. The supernatant was collected and stored at −80°C. HUVECs were cultured in EGM-2 media; only passage numbers 2–5 of HUVECs were used in this study. Cells were all maintained in a 5% CO_2 humidified atmosphere at 37°C.

2.3. Preparation of BSA-conjugated CLA isomer

A total of 500 mmol/L 9*E*,11*E*-CLA was prepared in ethanol, and aliquots were stored at −80°C until they were ready to conjugate with BSA. Thirty-two microliters of the stock 9*E*,11*E*-CLA in ethanol was transferred to a brown glass vial and dried with argon gas, while an equal volume of ethanol in another brown glass vial was dried for a vehicle control. One hundred thirty-two microliters of 0.15 mol/L KOH was added to both vials and vortexed under argon and was then incubated for 1 h at 70°C. At the end of the incubation, 2 ml of filter sterilized BSA in PBS (1 mmol/L) was added to the vehicle control and CLA isomer to make a final fatty acid concentration of 8 mmol/L. The pH level was adjusted to 7.0–7.5. BSA-conjugated CLA isomer and its BSA control were stored at −20°C until use.

2.4. RNA isolation and real-time PCR

Total RNA was isolated using Tri-Reagent (Sigma) according to the manufacturer's instructions. Reverse transcription was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the provider's instruction. Real-time PCR was performed as described previously [21]. Real-time PCR primers are shown in Table 1. The housekeeping gene, β-actin or 18S, was used to normalize all the tested genes. Data are shown as mean±S.E.M. with at least three independent experiments with triplicate samples.

2.5. Quantification of VCAM-1 by ELISA

VCAM-1 ELISA was performed based on the work of Lemaire et al. [22], with some modifications. Briefly, HUVECs (25,000 cells/well) were plated in a 96-well plate and allowed to recover overnight. On the following day, the cells were treated with either BSA vehicle control or 9*E*,11*E*-CLA as indicated for 24 h with or without TNF-α activation (2 ng/ml) for the last 4 h. At the end of the 24-h incubation, the media were removed and washed with PBS once. The primary antibody, 50 μl/well of 2.5-μg/ml human monoclonal VCAM-1 antibody in PBS containing 1% BSA and 0.01% NaN_3, was added to the plate and incubated at 4°C for 1 h. The cells were washed four times with PBS containing 1% BSA and then incubated with peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G diluted 1:500 in PBS containing 1% BSA at 4°C for 1 h. After the secondary antibody incubation, the cells were washed four times with PBS. One hundred microliters of fresh SIGMA FAST™ OPD was added to detect VCAM-1 in HUVECs at 490 nm.

2.6. Adhesion assay

Adhesion assay was performed to measure the cell-to-cell interaction between macrophages and ECs [23–25]. Briefly, HUVECs (25,000 cells/well) were plated in a 96-well plate and incubated overnight. On the following day, the cells were treated with either BSA vehicle control or 9*E*,11*E*-CLA as indicated for 24 h with or without 2 ng/ml of TNF-α for the last 4 h. RAW cells or stable RAW cells were fluorescently labeled with BCECF-AM (1 μM final concentration) in PBS by incubating them at 37°C for 45 min. The RAW cells were incubated with HUVECs at 37°C for 1 h. At the end of the 1-h incubation, unattached RAW cell lines were removed by washing twice with PBS. One hundred microliters of lysis buffer (10×: 50 μM of Tris buffer with 0.1% of SDS at pH 7.4) was added, and the fluorescence intensity was measured on excitation at 480 nm and emission at 530 nm.

2.7. Statistics

Data were normalized and equal variance was tested with Minitab (State College, PA, USA). The normalized data were analyzed by one-way analysis of variance. Tukey's family error rate was used for a one-way multiple comparison ($P<.05$). Values with an asterisk are different from the vehicle-treated control at the $P<.05$ level.

3. Results

3.1. 9E,11E-CLA inhibits initial atherogenesis-related genes in HUVECs

To determine the effects of 9*E*,11*E*-CLA on atherogenesis-related genes in ECs, we examined mRNA levels of ICAM-1, VCAM-1, monocyte chemoattractant protein-1 (MCP-1), C–C motif chemokine receptor-2 (CCR-2), E-selectin and P-selectin. HUVECs were treated with 100 μM

Table 1
List of real-time PCR primers

Primer name	Sequence
Human ICAM-1 forward	ACTCAGCGGTCATGTCTGGAC
Human ICAM-1 reverse	GGCATAGCTTGGGCATATTCC
Human VCAM-1 forward	AGTGGTGGCCTCGTGAATG
Human VCAM-1 reverse	CACGCTAGGAACCTTGCAGC
Human MCP-1 forward	ATAGCAGCCACCTTCATTCC
Human MCP-1 reverse	TGCACTGAGATCTTCCTATTGG
Human E-selectin forward	TCCTATTCCAGCCTGCAATGT
Human E-selectin reverse	AACCCATTGGCTGGATTTGTC
Human P-selectin forward	AGGAGAGTGCCTCGAGACCA
Human P-selectin reverse	TCCAGGGTAACAGGAGCAGGT
Human CCR-2 forward	GGACGCATTTCCCCAGTACA
Human CCR-2 reverse	CCGAGAACGAGATGTGGACA

9*E*,11*E*-CLA for 24 h with or without TNF-α activation for the last 4 h. Fig. 1 shows that the atherogenic target genes induced by TNF-α in ECs are decreased by 9*E*,11*E*-CLA. ICAM-1, VCAM-1 and CCR-2 mRNA levels are induced to 185-, 1330- and 11-fold, respectively, by TNF-α activation, but their induced levels are significantly attenuated by 40%, 95% and 70%, respectively, with 9*E*,11*E*-CLA ($P<.05$). 9*E*,11*E*-CLA also reduces TNF-α-induced MCP-1 (15-fold) and E-selectin (330-fold) mRNA levels by 70% and 66%, respectively. In addition, 9*E*,11*E*-CLA reduced basal levels of E-selectin, P-selectin and MCP-1. P-selectin mRNA levels are not significantly increased by TNF-α, although 9*E*,11*E*-CLA decreases its mRNA levels by 90% and 85% with and without TNF-α, respectively. P-selectin is immediately induced and peaked at 10 min after TNF-α activation, and it quickly declines after 3 h [26,27], which may explain the lack of its induction in this present study.

3.2. 9E,11E-CLA inhibits TNF-α-induced VCAM-1 levels in a dose-dependent manner in HUVECs

Among six genes tested, VCAM-1 showed the most significant reduction by 9*E*,11*E*-CLA in its mRNA levels (Fig. 1). Alteration of VCAM-1 protein levels was measured by ELISA. Fig. 2 shows that the alteration of VCAM-1 mRNA accumulation corresponds to the protein

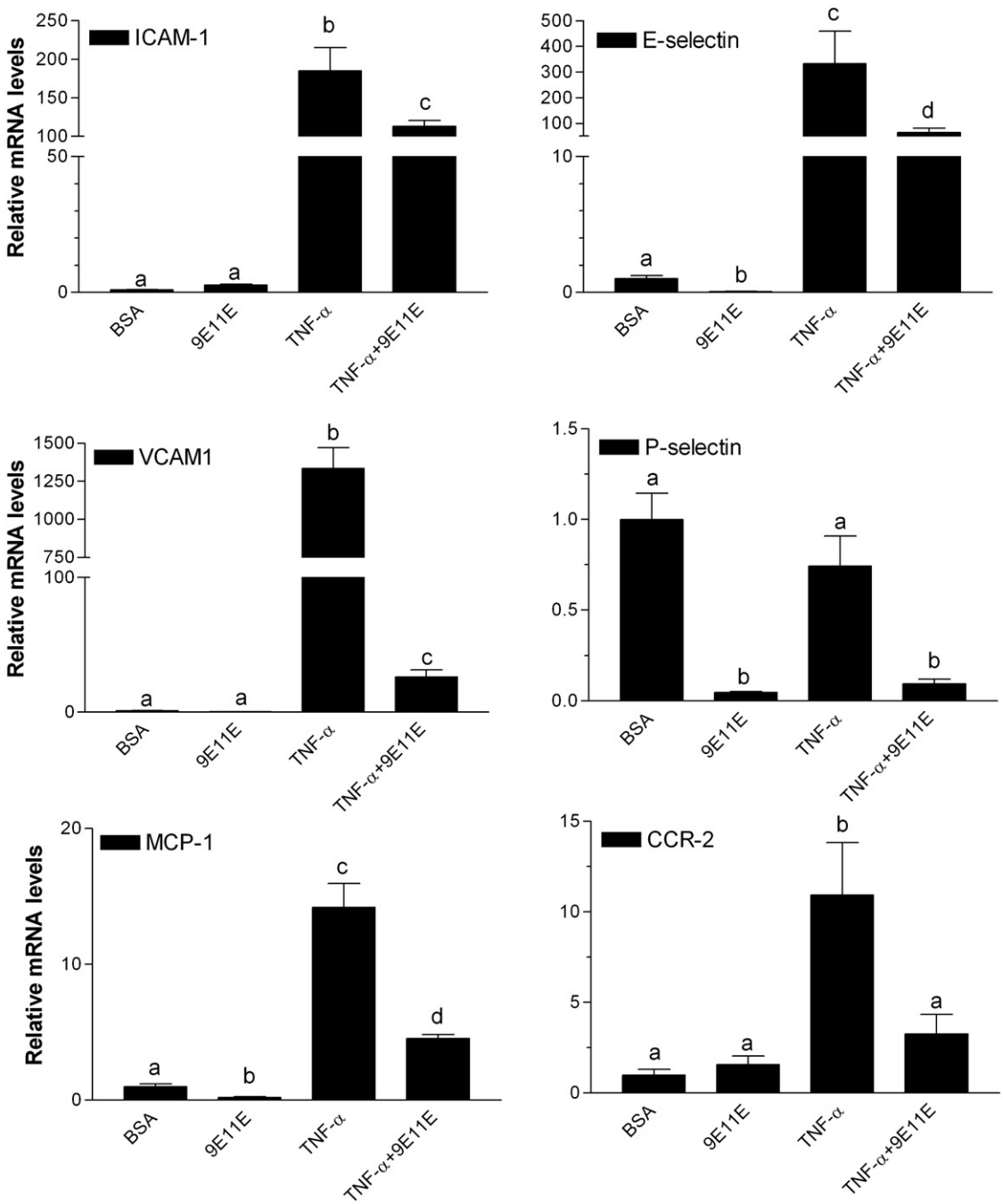

Fig. 1. 9*E*,11*E*-CLA attenuates mRNA levels of adhesion molecules in HUVECs. HUVECs were treated with vehicle control or 100 μM 9*E*,11*E*-CLA for 24 h with or without TNF-α (2 ng/ml) stimulation. Total RNA was isolated and real-time PCR was performed as described in Section 2. Data are from two independent experiments with triplicate samples. Significant difference is indicated by different letters ($P<.05$).

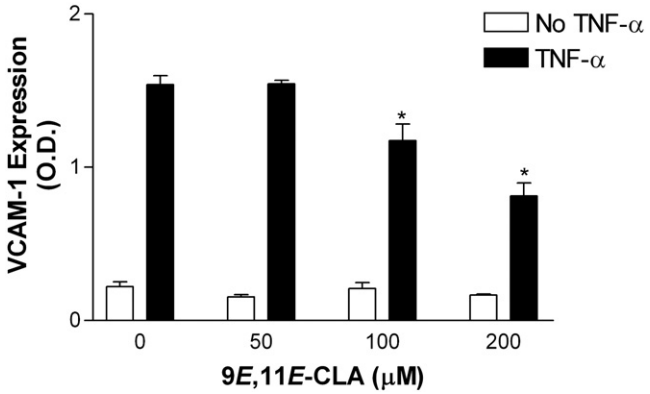

Fig. 2. 9*E*,11*E*-CLA inhibits VCAM-1 expression in a dose-dependent manner in HUVECs. HUVECs were treated with different concentrations of 9*E*,11*E*-CLA for 24 h with or without 2 ng/ml of TNF-α stimulation as described in Section 2. HUVECs were washed two times with PBS and VCAM-1 ELISA was performed as described in Section 2. Data represent two independent experiments with triplicate samples. Asterisks indicate a significant difference compared with vehicle control with TNF-α activation ($P<.05$).

levels of this gene. Without TNF-α activation, there was no significant change in VCAM-1 protein levels by 9*E*,11*E*-CLA in HUVECs. 9*E*,11*E*-CLA decreases TNF-α-induced VCAM-1 expression in a dose-dependent manner.

3.3. 9E,11E-CLA attenuates the adhesion activity of macrophage cells to ECs

9*E*,11*E*-CLA decreases the atherogenesis-related genes that are involved in the initiation of the development of a fatty streak lesion in HUVECs. To determine whether the changes of the atherogenesis-related genes by the CLA isomer affect adhesion activity, we performed adhesion assay as described in Section 2. 9*E*,11*E*-CLA-treated HUVECs with TNF-α activation have less macrophage adhesion (Fig. 3). TNF-α activation increased the cell-to-cell adhesion activity to 4.5-fold compared with the non-TNF-α-activated group. The induced cell-to-cell adhesion activity is inhibited by 9*E*,11*E*-CLA starting from 100 μM concentration in a dose-dependent manner. These results suggest that the changes of adhesion molecules by 9*E*,11*E*-CLA in HUVECs affect the functional level of the cell-to-cell interaction. There was no sign of overt toxicity with 9*E*,11*E*-CLA treatment up to 200 μM, as determined by trypan blue exclusion (data not shown).

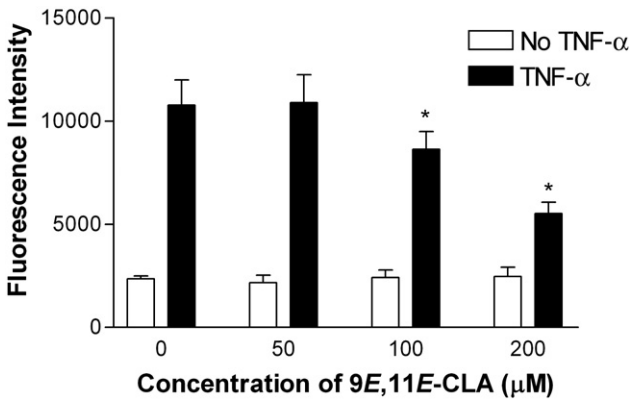

Fig. 3. 9*E*,11*E*-CLA reduces the adhesion activity of macrophages and ECs. HUVECs were treated with different concentrations of 9*E*,11*E*-CLA as indicated for 24 h with or without 2 ng/ml of TNF-α activation for the last 4 h. BCECF-AM-labeled RAW cells were incubated on the treated HUVECs for 1 h at 37°C. After washing with PBS twice, lysis buffer was added to each well. Fluorescence intensity was measured on excitation at 480 nm and emission at 530 nm. Data represent three independent experiments with triplicate samples. Asterisks indicate a significant difference compared with vehicle control with TNF-α activation ($P<.05$).

3.4. IL-1Ra alone is not enough to change the adhesion activity of macrophage cells and ECs

Through the use of siRNA in RAW cells, evidence supports that IL-1Ra contributes to the 9*E*,11*E*-CLA-dependent decrease in pro-inflammatory cytokines, IL-1α, IL-1β and IL-6 [16]. Additionally, IL-1Ra$^{-/-}$ mice accumulate inflammatory mononuclear cells and thickened intima by neointimal hyperplasia [28]. Thus, IL-1Ra is also a crucial regulator in vascular biology. We hypothesized that IL-1Ra affects the cell-to-cell interaction adhesion activity; however, 9*E*,11*E*-CLA does not significantly induce IL-1Ra levels in HUVECs (data not shown). Fig. 4 shows that mouse recombinant IL-1Ra (rIL-1Ra) does not alter the adhesion activity of macrophage cells or ECs. The amount of IL-1Ra protein produced when RAW cells were treated with 9*E*,11*E*-CLA is approximately 20–30 ng/ml. Within that range of rIL-1Ra, there is no significant adhesion activity change when it was added to either HUVECs (Fig. 4A) or RAW (Fig. 4B). These data suggest that increase of

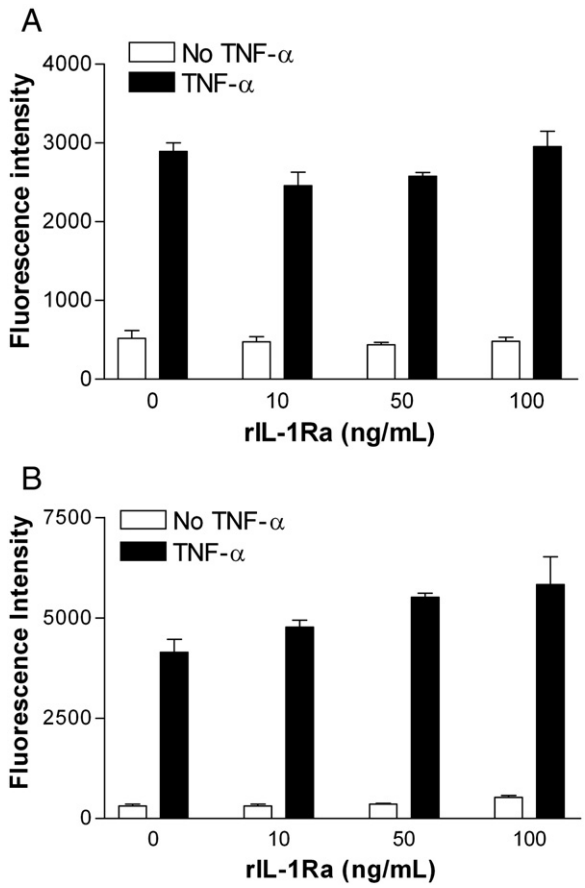

Fig. 4. rIL-1Ra does not alter the adhesion activity of HUVECs and RAW cells. (A) HUVECs were treated with different concentrations of rIL-1Ra for 24 h as indicated. At the end of the 24-h incubation period, TNF-α (2 ng/ml) was or was not added to activate HUVECs for the last 4 h. RAW cells were labeled with BCECF-AM as described in Section 2. HUVECs and RAW cells were incubated together for 1 h, and fluorescence intensity was measured on excitation at 480 nm and emission at 530 nm. (B) RAW cells were treated with different concentrations of rIL-1Ra for 24 h and then labeled with BCECF-AM. HUVECs were activated with TNF-α (2 ng/ml) for 4 h before the adhesion assay began. At the end of the 1-h incubation, fluorescence intensity was measured on excitation at 480 nm and emission at 530 nm. Data represent two independent experiments with triplicate samples.

IL-1Ra by itself cannot be enough to explain the effect of 9*E*,11*E*-CLA on adhesion.

3.5. Changes in adhesion activity of macrophage cells by 9E,11E-CLA may be indirectly regulated by IL-1Ra induction

Stable cell lines expressing low IL-1Ra were generated in RAW 264.7 cells previously [29]. The adhesion activity of IL-1Ra-deficient RAW 264.7 cells (siIL-1Ra RAW) cells was higher than that of control stable cells (si-control RAW) and wild-type RAW cells to TNF-α-activated HUVECs (Fig. 5A). The basal binding activity of siIL-1Ra RAW cells to non-activated HUVECs was also higher than that of si-control RAW to HUVECs, suggesting that siIL-1Ra RAW cells have a higher adhesion activity to HUVECs compared with its control RAW cells to HUVECs. As previously shown, the induced IL-1Ra levels by 9*E*,11*E*-CLA are abolished with LY294002 and rapamycin in RAW cells, suggesting that the IL-1Ra induction by 9*E*,11*E*-CLA is regulated via the PI3K/mTOR pathway. Fig. 5B shows that RAW cells co-treated with LY294002 or rapamycin have a higher adhesion activity to TNF-α-activated HUVECs than RAW cells treated with 9*E*,11*E*-CLA alone. Without TNF-α activation, there is no change observed among treatment groups. This result confirms the importance of IL-1Ra regulation by 9*E*,11*E*-CLA in altering the cell-to-cell adhesion activity in macrophage cells to HUVECs.

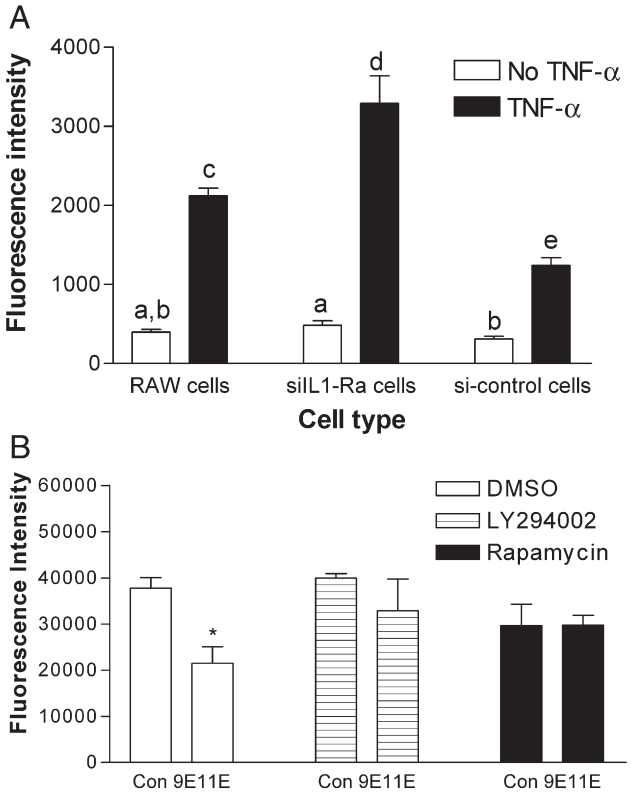

Fig. 5. IL-1Ra deficiency in RAW cells affects the adhesion activity of RAW cells, and LY294002 and mTOR reverse the decrease in the adhesion activity of 9*E*,11*E*-CLA. (A) Three cell lines, RAW cells (wild type), siIL-1Ra RAW cells and si-control RAW cells, were labeled with BCECF-AM. HUVECs were plated in a 96-well plate and on the following day were activated with 2 ng/ml of TNF-α for 4 h. The labeled RAW cells were plated on HUVECs for 1 h at 37°C. (B) RAW cells were plated and starved for 24 h. On the following day, the RAW cells were pre-treated with DMSO, LY294002 (14 μM) or rapamycin (20 nM) for 30 min and co-treated with 100 μM 9*E*,11*E*-CLA for 10 h. The treated RAW cells were harvested and labeled with BCECF-AM. HUVECs were plated and activated with 2 ng/ml of TNF-α for 4 h. The labeled RAW cells were incubated with HUVECs for 1 h at 37°C. Fluorescence intensity was measured on excitation at 480 nm and emission at 530 nm. Data represent two independent experiments with triplicate samples. A significant difference is indicated as a different letter or asterisk ($P<.05$).

3.6. Macrophage-secreted factors from siIL-1Ra RAW and si-control RAW cells regulate atherogenesis-related gene expression differently in HUVECs

Whether 9*E*,11*E*-CLA-induced factors from macrophages could affect adhesion molecules in HUVECs was examined. Since the IL-1Ra siRNA cell lines were utilized in this study, the role of this endogenous anti-inflammatory molecule on cell-to-cell communication was also explored. The conditioned medium from each RAW cell line was collected after LPS or LPS plus 9*E*,11*E*-CLA treatment (Fig. 6). mRNA levels of ICAM-1, VCAM-1, MCP-1 and E-selectin induced by siIL-1Ra RAW LPS-conditioned media were significantly higher than those of the genes induced by si-control RAW LPS-conditioned media in HUVECs. LPS plus 9*E*,11*E*-CLA-conditioned media from siIL-1Ra RAW cells did not decrease the induced mRNA levels of ICAM-1, VCAM-1, MCP-1 and E-selectin; however, LPS plus 9*E*,11*E*-CLA-conditioned media from si-control RAW cells significantly decreased these transcripts in HUVECs. mRNA levels of CCR-2 and P-selectin did not show a significant difference between HUVECs treated with siIL-1Ra RAW-conditioned media and those treated with si-control RAW-conditioned media. However, LPS plus 9*E*,11*E*-CLA media from si-control RAW cells increased P-selectin mRNA levels to 2.5-fold in HUVECs compared with its levels in HUVEC-treated LPS-conditioned media from si-control RAW. These data show that 9*E*,11*E*-CLA treatment of macrophages produces signaling molecules that affect gene expression in the EC. In addition, a subset of the genes affected in this manner results from IL-1Ra-dependent events in the macrophage.

4. Discussion

Atherosclerosis has been defined as an inflammatory disease [3], although hypercholesterolemia is a contributory risk factor. The anti-atherogenic effects of CLA vary in in vitro and in vivo models (reviewed by Wahle et al. [7], Wang & Jones [30] and Nagao & Yanagita [31]). CLA reduced the risk of the development of atherosclerosis in rabbits and hamsters [8,9,32]. Toomey et al. showed that CLA (9*Z*,11*E*-CLA/10*E*,12*Z*-CLA, 80:20 blend) fed to apolipoprotein E-deficient mice suppresses atherosclerotic lesions by attenuating pro-inflammatory genes, such as matrix metalloproteinase-9 and platelet endothelial cell adhesion molecule-1, and enhances apoptotic genes, such as caspase-3 [33]. On the other hand, Schleser et al. showed no effect of 9*Z*,11*E*-CLA and 10*E*,12*Z*-CLA to reduce adhesion molecules in human aortic ECs [14,15]. Nugent et al. reported that CLA did not affect prostaglandin E_2 and ICAM-1 in healthy humans [14]. Another human CLA study reported that 2-month CLA supplementation had no change on lipid profiles, such as the levels of cholesterol, LDL, HDL and triglycerides, and platelet aggregation in healthy subjects [34,35]. Munday et al. reported that CLA increases fatty streak development in the aorta in mice fed a high-fat diet [12]. This discrepancy of atherogenesis-related effects of CLA may be due to the relative amounts and composition of the CLA used in the study. Minor CLA isomers, such as 9*E*,11*E*-CLA, have not been examined for their role in inhibiting atherogenesis.

9*E*,11*E*-CLA has an anti-inflammatory effect by enhancing IL-1Ra while attenuating pro-inflammatory cytokines such as IL-1α, IL-1β and IL-6 [16]. In addition, the induction of IL-1Ra by the CLA isomer is diminished by PI3K (LY294002) and mTOR (rapamycin) inhibitors [29]. It was hypothesized that the anti-inflammatory effects of 9*E*,11*E*-CLA, in particular, enhancing IL-1Ra, may reduce pro-atherogenic factors in ECs. 9*E*,11*E*-CLA attenuates mRNA levels of pro-atherogenic genes stimulated with TNF-α, such as adhesion molecules in HUVECs,

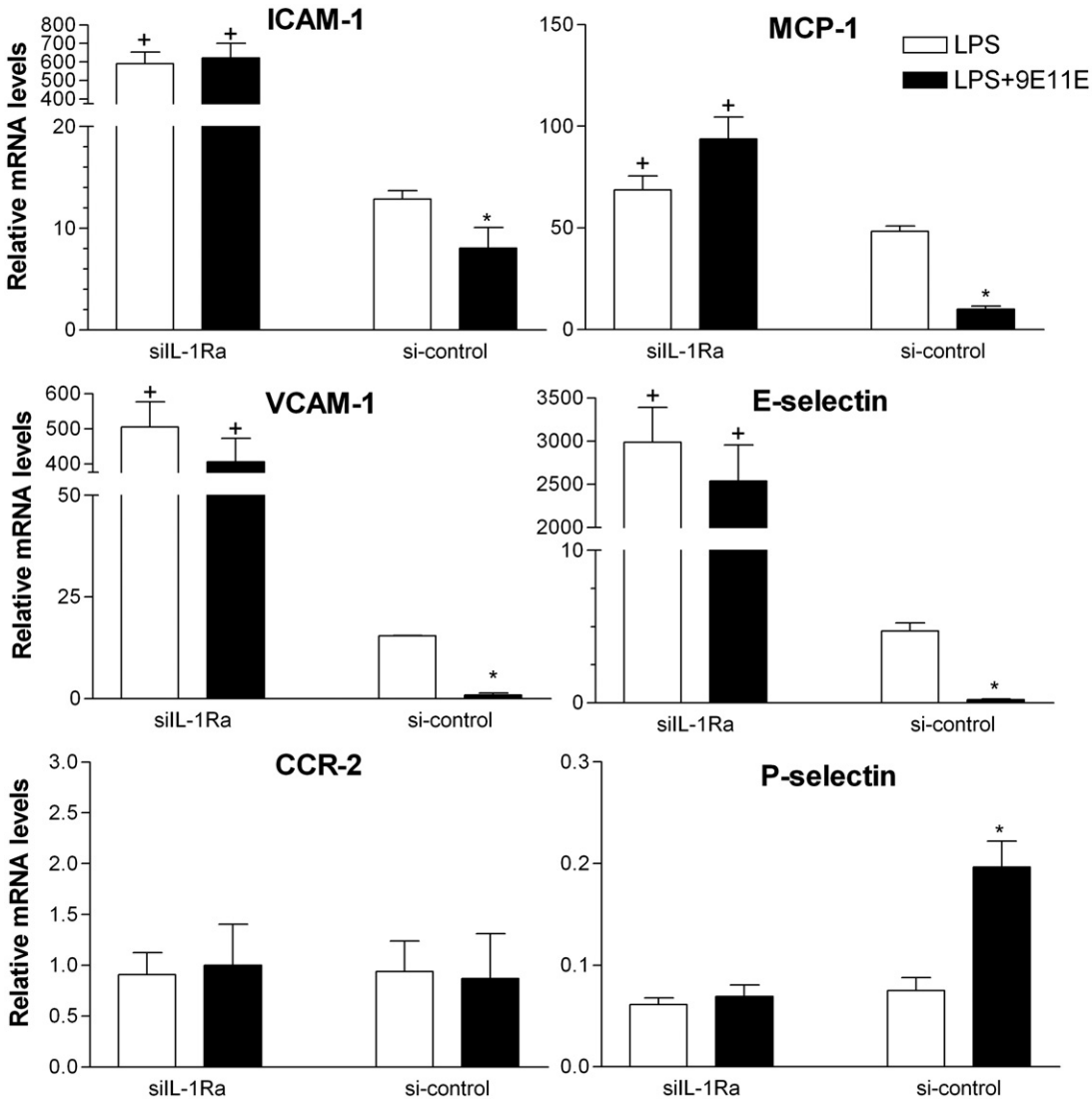

Fig. 6. Conditioned media from siIL-1Ra RAW cell line and si-control RAW cell line regulate atherogenesis-related genes in HUVECs. siIL-1Ra RAW- and si-control RAW-conditioned media were collected as described in Section 2. Twenty percent of siIL-1Ra RAW or si-control RAW-conditioned media was treated in HUVECs for 24 h. siIL-1Ra RAW-conditioned media collected after BSA without LPS were used as a negative control set as 1. RNA was isolated and real-time PCR was performed as described in Section 2. Data represent two independent experiments with triplicate samples. The asterisk indicates a significant difference compared with the LPS group, while the plus mark indicates a significant difference compared with the LPS group in the si-control media-treated group ($P<.05$).

and functional levels of the adhesion activity of HUVECs and RAW cells. Adhesion molecules mediate the cell-to-cell interactions that are involved in the initial event of atherosclerotic lesions. There are four major families of adhesion molecules: selectins, selectin ligands, integrins and the Ig family of proteins [36,37]. The selectins and selectin ligands are involved in leukocyte rolling, and the integrins and members of the Ig superfamily are involved in leukocyte adhesion and transmigration. The TNF-α-induced P-selectin and E-selectin are reduced by 9*E*,11*E*-CLA, although no robust induction of P-selectin was observed in this study. This may be explained by the different regulation patterns of the two selectins. The production of E-selectin is regulated by the expression of P-selectin, which was induced almost immediately (within minutes of cytokine recognition). On the other hand, E-selectin expression peaks around 6–12 h after cytokine recognition [38]. TNF-α was added for the last 4 h of the total 24-h treatment with 9*E*,11*E*-CLA in this study, thus missing the immediate induction of P-selectin. Double knockout of MCP-1 and LDL receptor in mice reduced lipid deposition in aortas (83%) and fewer macrophages in their aortic walls [39]. MCP-1 levels were decreased by 9*E*,11*E*-CLA. MCP-1 binds to CCR-2, and CCR-2-deficient mice have a phenotype similar to that of MCP-1-deficient mice [40]. Anti-MCP-1 gene therapy had beneficial effects on atherosclerosis, such as stabilizing a plaque prior to rupture [41].

VCAM-1 is highly expressed in endothelium in fibrous and lipid-containing plaques [42], suggesting its importance and correlation in atherosclerosis. Protein levels of VCAM-1, one of the most important adhesion molecules in the leukocyte recruitment to atherosclerotic lesions [36,37], were decreased in HUVECs by 9*E*,11*E*-CLA in a dose-dependent manner. The adherence of macrophages to 9*E*,11*E*-CLA-treated HUVECs decreased in a dose-dependent manner. 9*E*,11*E*-CLA-treated macrophages bind less to laminin compared with vehicle controls, and the same pattern is shown in LPS-challenged groups. The decrease in adhesion activity of RAW cells treated with 9*E*,11*E*-CLA to TNF-α-activated HUVECs was diminished with the PI3K and mTOR kinase inhibitors LY294002 and rapamycin. These treatments also decreased IL-1Ra induction by this CLA isomer in RAW cells, suggesting a potential involvement of IL-1Ra derived from RAW cells in adhesion activity [29].

IL-1Ra is expressed in human ECs and atherosclerotic lesions [43,44]; however, 9*E*,11*E*-CLA does not significantly increase IL-1Ra in HUVECs (data not shown). siIL-1Ra RAW cells were utilized and conditioned RAW media experiments were performed in HUVECs to investigate the effects of IL-1Ra produced by 9*E*,11*E*-CLA in macrophages to ECs. Co-culture study and conditioned media study with RAW 264.7 cells and HUVECs were previously used by others [45,46]. siIL-1Ra RAW cells bind more to TNF-α-activated HUVECs compared with control RAW cells. Conditioned media of siIL-1Ra RAW LPS overall increase the mRNA levels of the adhesion molecules in HUVECs compared with their levels in si-control-treated HUVECs. Although the major difference between the two conditioned media is the level of IL-1Ra, it is worthwhile to note that the conditioned media contain LPS and 9*E*,11*E*-CLA, as well as various unknown factors secreted by RAW cells. The comparison of siIL-1Ra RAW-conditioned media and si-control RAW-conditioned media indicates a dramatic difference of macrophage-secreted factors from the two stable cell lines. Overall, siIL-1Ra RAW-conditioned media-treated HUVECs express significantly higher levels of ICAM-1, VCAM-1, MCP-1 and E-selectin with no beneficial effect of 9*E*,11*E*-CLA. Surprisingly, rIL-1Ra treatment of HUVECs and RAW cells showed that this molecule alone does not alter the adhesion activity of both cell lines in a range of IL-1Ra's secreted by RAW cells upon 9*E*,11*E*-CLA treatment. This result suggests that factors secreted from siIL-1Ra RAW cells enhance the adhesion molecules and that 9*E*,11*E*-CLA's effect to alter the adhesion activity may be secondary to IL-1Ra-level alteration.

Although 9*E*,11*E*-CLA decreased adhesion molecules and activity in HUVECs, mRNA levels of ADAMTS1 (a disintegrin-like and metallopeptidase-1) were enhanced by this particular CLA isomer in RAW cells [16]. ADAMTS1 is an extracellular metalloproteinase known to participate in a variety of biological processes that include inflammation, angiogenesis and development of the urogenital system [47,48]. ADAMTS1 weakens plaque in atherosclerosis lesion; therefore, it is an important factor in the later stage of atherosclerosis fatty streak lesion formation, which can be a potential negative effect of 9*E*,11*E*-CLA in atherogenesis.

Taken together, the data show that 9*E*,11*E*-CLA attenuates adhesion molecules and adhesion activity in HUVECs stimulated by TNF-α. Not only IL-1Ra but also other secretory factors by 9*E*,11*E*-CLA in macrophage cells play a role to regulate the adhesion activity. With this in vitro result of 9*E*,11*E*-CLA's potential health benefits, an in vivo study may bring us more interesting insights into this unique CLA isomer at biological and physiological levels.

References

[1] Lusis AJ. Atherosclerosis. Nature 2000;407:233–41.
[2] Weissberg PL. Atherogenesis: current understanding of the causes of atheroma. Heart 2000;83:247–52.
[3] Ross R. Atherosclerosis — an inflammatory disease. N Engl J Med 1999;340:115–26.
[4] Glass CK, Witztum JL. Atherosclerosis: the road ahead. Cell 2001;104:503–16.
[5] Pariza MW, Ha YL. Conjugated dienoic derivatives of linoleic acid: a new class of anticarcinogens. Med Oncol Tumor Pharmacother 1990;7:169–71.
[6] Pariza MW, Ha YL. Newly recognized anticarcinogenic fatty acids. Basic Life Sci 1990;52:167–70.
[7] Wahle KW, Heys SD, Rotondo D. Conjugated linoleic acids: are they beneficial or detrimental to health? Prog Lipid Res 2004;43:553–87.
[8] Nicolosi RJ, Rogers EJ, Kritchevsky D, Scimeca JA, Huth PJ. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery 1997;22:266–77.
[9] Kritchevsky D, Tepper SA, Wright S, Czarnecki SK, Wilson TA, Nicolosi RJ. Conjugated linoleic acid isomer effects in atherosclerosis: growth and regression of lesions. Lipids 2004;39:611–6.
[10] Song HJ, Sneddon AA, Heys SD, Wahle KW. Induction of apoptosis and inhibition of NF-kappaB activation in human prostate cancer cells by the *cis*-9, *trans*-11 but not the *trans*-10, *cis*-12 isomer of conjugated linoleic acid. Prostate 2006;66:839–46.
[11] Bhattacharya A, Banu J, Rahman M, Causey J, Fernandes G. Biological effects of conjugated linoleic acids in health and disease. J Nutr Biochem 2006.
[12] Munday JS, Thompson KG, James KA. Dietary conjugated linoleic acids promote fatty streak formation in the C57BL/6 mouse atherosclerosis model. Br J Nutr 1999;81:251–5.
[13] Chen BQ, Yang YM, Wang Q, Gao YH, Liu JR, Zhang JS, et al. Effects of c9,*t*11-conjugated linoleic acid on adhesion of human gastric carcinoma cell line SGC-7901. World J Gastroenterol 2004;10:1392–6.
[14] Nugent AP, Roche HM, Noone EJ, Long A, Kelleher DK, Gibney MJ. The effects of conjugated linoleic acid supplementation on immune function in healthy volunteers. Eur J Clin Nutr 2005;59:742–50.
[15] Schleser S, Ringseis R, Eder K. Conjugated linoleic acids have no effect on TNFalpha-induced adhesion molecule expression, U937 monocyte adhesion, and chemokine release in human aortic endothelial cells. Atherosclerosis 2006;186:337–44.
[16] Lee Y, Thompson JT, de Lera AR, Vanden Heuvel JP. Isomer-specific effects of conjugated linoleic acid on gene expression in RAW 264.7. J Nutr Biochem 2008, doi:10.1016/j.jnutbio.2008.07.013. Epub ahead of press.
[17] Isoda K, Ohsuzu F. The effect of interleukin-1 receptor antagonist on arteries and cholesterol metabolism. J Atheroscler Thromb 2006;13:21–30.
[18] Kusuhara M, Isoda K, Ohsuzu F. Interleukin-1 and occlusive arterial diseases. Cardiovasc Hematol Agents Med Chem 2006;4:229–35.
[19] Francis SE, Camp NJ, Dewberry RM, Gunn J, Syrris P, Carter ND, et al. Interleukin-1 receptor antagonist gene polymorphism and coronary artery disease. Circulation 1999;99:861–6.
[20] Yu Y, Correll PH, Vanden Heuvel JP. Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. Biochim Biophys Acta 2002;1581:89–99.
[21] Coleman JD, Prabhu KS, Thompson JT, Reddy PS, Peters JM, Peterson BR, et al. The oxidative stress mediator 4-hydroxynonenal is an intracellular agonist of the nuclear receptor peroxisome proliferator-activated receptor-beta/delta (PPAR-beta/delta). Free Radic Biol Med 2007;42:1155–64.
[22] Lemaire S, Lizard G, Monier S, Miguet C, Gueldry S, Volot F, et al. Different patterns of IL-1beta secretion, adhesion molecule expression and apoptosis induction in human endothelial cells treated with 7alpha-, 7beta-hydroxycholesterol, or 7-ketocholesterol. FEBS Lett 1998;440:434–9.
[23] Vaporciyan AA, Jones ML, Ward PA. Rapid analysis of leukocyte-endothelial adhesion. J Immunol Methods 1993;159:93–100.
[24] Sakamoto K, Ito Y, Mori T, Sugimura K. Interaction of human lactoferrin with cell adhesion molecules through RGD motif elucidated by lactoferrin-binding epitopes. J Biol Chem 2006;281:24472–8.
[25] Kim JD, Liu L, Guo W, Meydani M. Chemical structure of flavonols in relation to modulation of angiogenesis and immune-endothelial cell adhesion. J Nutr Biochem 2006;17:165–76.
[26] Gotsch U, Jager U, Dominis M, Vestweber D. Expression of P-selectin on endothelial cells is upregulated by LPS and TNF-alpha in vivo. Cell Adhes Commun 1994;2:7–14.
[27] Weller A, Isenmann S, Vestweber D. Cloning of the mouse endothelial selectins. Expression of both E- and P-selectin is inducible by tumor necrosis factor alpha. J Biol Chem 1992;267:15176–83.
[28] Isoda K, Shiigai M, Ishigami N, Matsuki T, Horai R, Nishikawa K, et al. Deficiency of interleukin-1 receptor antagonist promotes neointimal formation after injury. Circulation 2003;108:516–8.
[29] Lee Y, Thompson JT, Vanden Heuvel JP. Increased expression of the endogenous anti-inflammatory factor IL-1 receptor antagonist (IL-1Ra) by 9*E*,11*E*-conjugated linoleic acid. J Nutr 2009 [submitted].
[30] Wang YW, Jones PJ. Conjugated linoleic acid and obesity control: efficacy and mechanisms. Int J Obes Relat Metab Disord 2004;28:941–55.
[31] Nagao K, Yanagita T. Conjugated fatty acids in food and their health benefits. J Biosci Bioeng 2005;100:152–7.
[32] Lee KN, Kritchevsky D, Pariza MW. Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis 1994;108:19–25.
[33] Toomey S, Harhen B, Roche HM, Fitzgerald D, Belton O. Profound resolution of early atherosclerosis with conjugated linoleic acid. Atherosclerosis 2006;187:40–9.
[34] Benito P, Nelson GJ, Kelley DS, Bartolini G, Schmidt PC, Simon V. The effect of conjugated linoleic acid on plasma lipoproteins and tissue fatty acid composition in humans. Lipids 2001;36:229–36.
[35] Benito P, Nelson GJ, Kelley DS, Bartolini G, Schmidt PC, Simon V. The effect of conjugated linoleic acid on platelet function, platelet fatty acid composition, and blood coagulation in humans. Lipids 2001;36:221–7.
[36] Krieglstein CF, Granger DN. Adhesion molecules and their role in vascular disease. Am J Hypertens 2001;14:44S–54S.
[37] Huo Y, Ley K. Adhesion molecules and atherogenesis. Acta Physiol Scand 2001;173:35–43.
[38] Leeuwenberg JF, Smeets EF, Neefjes JJ, Shaffer MA, Cinek T, Jeunhomme TM, et al. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. Immunology 1992;77:543–9.
[39] Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, et al. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 1998;2:275–81.
[40] Kurihara T, Warr G, Loy J, Bravo R. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J Exp Med 1997;186:1757–62.
[41] Inoue S, Egashira K, Ni W, Kitamoto S, Usui M, Otani K, et al. Anti-monocyte chemoattractant protein-1 gene therapy limits progression and destabilization of established atherosclerosis in apolipoprotein E-knockout mice. Circulation 2002;106:2700–6.

[42] O'Brien KD, Allen MD, McDonald TO, Chait A, Harlan JM, Fishbein D, et al. Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. Implications for the mode of progression of advanced coronary atherosclerosis. J Clin Invest 1993;92:945–51.
[43] Dewberry R, Holden H, Crossman D, Francis S. Interleukin-1 receptor antagonist expression in human endothelial cells and atherosclerosis. Arterioscler Thromb Vasc Biol 2000;20:2394–400.
[44] Gottsater A, Forsblad J, Matzsch T, Persson K, Ljungcrantz I, Ohlsson K, et al. Interleukin-1 receptor antagonist is detectable in human carotid artery plaques and is related to triglyceride levels and *Chlamydia pneumoniae* IgA antibodies. J Intern Med 2002;251:61–8.
[45] Peng HB, Spiecker M, Liao JK. Inducible nitric oxide: an autoregulatory feedback inhibitor of vascular inflammation. J Immunol 1998;161:1970–6.
[46] Spiecker M, Peng HB, Liao JK. Inhibition of endothelial vascular cell adhesion molecule-1 expression by nitric oxide involves the induction and nuclear translocation of IkappaBalpha. J Biol Chem 1997;272:30969–74.
[47] Kuno K, Okada Y, Kawashima H, Nakamura H, Miyasaka M, Ohno H, et al. ADAMTS-1 cleaves a cartilage proteoglycan, aggrecan. FEBS Lett 2000;478: 241–5.
[48] Torres-Collado AX, Kisiel W, Iruela-Arispe ML, Rodriguez-Manzaneque JC. ADAMTS1 interacts with, cleaves, and modifies the extracellular location of the matrix inhibitor tissue factor pathway inhibitor-2. J Biol Chem 2006;281:17827–37.