Effect of palmitic acid and linoleic acid on expression of ICAM-1 and VCAM-1 in human bone marrow endothelial cells (HBMECs)

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Abstract

Introduction: The amount and type of fatty acids (FAs) in the diet influence the risk of atherosclerosis. Palmitic acid and linoleic acid exist at high levels in Iranian edible oils. In this study, we investigated the effect of palmitic acid and linoleic acid on expression of soluble and cell-associated forms of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in human bone marrow endothelial cells (HBMECs).

Material and methods: The endothelial cells were induced with bacterial lipopolysaccharide (LPS) or tumor necrosis factor α (TNF-α), and thereafter incubated with palmitic or linoleic acid. The level of soluble and cell-associated VCAM-1 and ICAM-1 were analyzed using ELISA and western blot.

Results: Our findings indicated that palmitic acid up-regulates the expression of ICAM-1 and VCAM-1 in HBMECs when these cells are induced with TNF-α or LPS. In addition, the results suggest that linoleic acid could sustain up-regulated ICAM-1 and VCAM-1 in activated endothelial cells.

Conclusions: Chronic activation of endothelial cells in the presence of palmitic and linoleic may account for pathogenesis of cardiovascular events. These findings provide further support for the detrimental effects of these fatty acids, especially palmitic acid, in promotion and induction of cardiovascular diseases which are prevalent in the Iranian population.

Key words: human bone marrow endothelial cells, intercellular adhesion molecule-1, fatty acid, vascular cell adhesion molecule-1.

Introduction

Research conducted by the Iranian Nutrition Institute (INI) showed that roughly 21% of the population's food intake results from the consumption of edible oils. If the intake of other fats contained in other food products are taken into account, this percentage would exceed 30%, the maximum recommended amount of consumption. Iran will experience a significant increase in both per capita and total consumption of edible oils and feed meals over the next 10 years [1]. Palmitic acid (C16:0) and linoleic acid (C18:2n6) are the most common saturated and polyunsaturated fatty acids consumed in the Iranian population, respectively [2]. The amount and type of fatty acids in the diet influence the risk of inflammatory disease and atherosclerosis [3, 4]. Fats rich in long-chain saturated fatty acids (SFA)

such as butter have been shown to exacerbate postprandial lipemic responses [5]. Current dietary recommendations for reducing cardiovascular disease (CVD) risk emphasize reducing the intake of SFA (mainly palmitic acid) by increasing dietary monounsaturated fatty acids (MUFA) [6, 7]. On the other hand, the findings regarding the detrimental effect of linoleic acid, n-6 polyunsaturated fatty acids (PUFA) abundant in many vegetable oils, on cardiovascular disease are controversial [8-12].

Atherosclerosis is thought to be initiated at critical sites of the arterial vasculature by a process of leukocyte adhesion to the vessel wall, sustained by the occurrence of active functional changes on the endothelial surface [13]. Normally, vascular endothelial cells have low adhesiveness for leukocytes. However, when stimulated, they express adhesion molecules at their surfaces responsible for firm attachment/activation of leukocytes and initiation of inflammation. The members of the immunoglobulin gene superfamily of adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), are among the most prominent adhesion molecules. These adhesion molecules interact with integrins at cellular surfaces and control the traffic of leukocytes between blood and tissues [14-17]. Human bone marrow endothelial cells (HBMECs), a representative of microvascular endothelial cells, synthesize the adhesion molecules to control the traffic of hematopoietic cells from and to the blood [18-20].

Previously, we reported that elaidic acid sustains ICAM-1 and VCAM-1 up-regulated by tumour necrosis factor α (TNF-α) or lipopolysaccharide (LPS) [21]. Herein, we have investigated the effect of palmitic acid and linoleic acid, the two most common fatty acids consumed in Iran, on expression of membrane and secreted forms of VCAM-1 and ICAM-1 in HBMECs, in order to determine the probable relationship between immoderate consumption of these FAs and high atherosclerosis prevalence in the Iranian population.

Material and methods

Chemicals and cells

Seventy five cm^2 culture flasks, culture microplates (NUNC, Roskilde, Denmark), trypsin/ EDTA solution, MCDB131, penicillin, streptomycin, amphotericin B, fetal bovine serum (FBS) (Gibco, New York, USA), free essential fatty acid – bovine serum albumin (FEFA-BSA), palmitic acid (C16), linoleic acid (C18:2n6) and human recombinant TNF-α (T 0157) were purchased from Sigma (St. Louis, MO, USA). LPS (31.6-25 Bacto) was purchased from DIFCO (Kansas, USA). Monoclonal antibodies against VCAM-1 (AHT0603) and ICAM-1 (AHS5444), ELISA-based kits (96 wells) for sICAM-1 (KHS4412/ KHS5411) and sVCAM-1(KHT0611) were purchased from Biosource (Invitrogen Corp. USA). Rabbit anti-mouse IgG (horseradish peroxidase conjugated) was prepared in our laboratory. The HBMECs were kindly provided by Dr. Manocher Mirshahi (College of Medical Sciences, Tarbiat Modares University, Tehran, Iran).

Endothelial cell culture

The HBMECs at the 25th passage were cultured in a complete medium containing MCDB131 supplemented with 10% FBS, 100 Iu/ml penicillin, 100 μg/ml streptomycin in 75 cm^2 culture flasks at 37°C and 5% CO_2. After reaching confluence, the cells were treated with trypsin-EDTA and plated to yield near-confluent cultures at the end of the experiment. The freshly plated cells were allowed to attach in standard growth medium for at least 48 h. The HBMECs were pretreated with TNF-α or LPS for induction of adhesion molecule expression, and then treated with palmitic or linoleic acid according to the experimental design (see below).

Experimental design

Cultured HBMECs were incubated with two different concentrations of TNF-α (0.01 and 0.001 μg/ml) and three different concentrations of LPS (0.1, 1 and 10 μg/ml) for 6, 12, 18 and 24 h to find optimal concentrations and incubation times of these stimulants. In separate experiments, the cultured HBMECs were also incubated with three different concentrations (10, 50 and 100 μM) of fatty acids (palmitic or linoleic acid) for 24, 48 and 72 h to find optimal concentrations of these fatty acids with minimal toxic effects. Palmitic and linoleic acid were converted to their potassium salts through the saponification method [22], then aliquoted under an N_2 stream, and stored at –80°C for no longer than 5 months before use. At the time of the experiments, fatty acids were dissolved in FEFA-BSA at the final desired concentrations. The fatty acid stock solutions were diluted in the medium to reach fatty acid concentrations of 10 μM to 100 μM with corresponding BSA concentrations of 2 μM to 20 μM (molar ratio of fatty acid to albumin was 5 : 1) [23]. Equivalent amounts of BSA alone were added to control plates. At the end of these periods, the protein pattern (SDS-PAGE) and parameters of cell viability (morphology, number and viability (trypan blue exclusion)) were assessed. The best concentrations with maximum viability and protein expression for each TNF-α, LPS and fatty acids were selected. All experiments were performed at least in triplicate.

Fatty acid treatment

Fatty acid (1000 μM)/BSA (200 mM) stock solutions were prepared by dissolving FEFA-BSA and

palmitic or linoleic acid (potassium salts) in normal saline (sodium chloride 0.9% w/v). The filter-sterilized fatty acids-BSA mixture (0.5 ml) was added to 4.5 ml of fresh MCDB131 medium supplemented with 10% fetal bovine serum (FBS) (final concentration of fatty acids 100 μM). The cells incubated in MCDB131 and BSA (20 mM) supplemented with 10% FCS in absence of fatty acids, TNF-α, or LPS were used as negative controls.

Detection of cell adhesion molecules

Assay of cell adhesion molecules was carried out by both ELISA and western blot analysis.

ELISA

The HBMECs were cultured in 75 cm^2 culture flasks and allowed to reach confluence. After adding the stimulants and fatty acids, soluble (in supernatant) and cell-associated (in cell lysate) forms of ICAM-1 and VCAM-1 were quantified using the ELISA kits, based on a sandwich format. For preparation of cell lysate, the endothelial cells were washed three times with PBS and solubilized in Tris-HCl buffer (20 mM, pH 8.0), containing 1% NP-40, 1% CHAPS, 50 mM NaCl, 10% glycerol, 2 mM EDTA and 1 mM PMSF overnight at 4°C. The cell lysate was centrifuged at 10 000 ×g for 10 min at 4°C and the clear supernatant was immediately used for determination of ICAM-1 and VCAM-1. The data presented are means of triplicate determinations.

Western blot analysis

The HBMECs were grown to confluence in 75 cm^2 culture flasks, gently washed twice with ice-cold Tris-HCl buffer (10 mM) containing 250 mM sucrose and then lysed in 0.5 ml of lysis buffer (20 mM Tris-HCl pH 7.4 containing 50 mM NaCl, 1% NP-40, 10% glycerol, 1 mM PMSF, 2 mM EDTA and 10 μg/ml aprotinin). After 10 min on ice, cell lysates were collected by centrifugation (14 000 ×g for 15 min at 4°C). The supernatant was boiled in SDS-PAGE sample buffer containing 10% SDS for 10 min and resolved in 12.5% polyacrylamide separating gels at 150 V. The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane using tank blotting [24]. The membranes were rinsed 3 times in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20, and then blocked in PBS-BSA 2% (w/v) for 2 h. The membranes were incubated overnight (12 h) in primary (mouse anti-human VCAM-1 and ICAM-1 monoclonal antibodies) and for 2 h in secondary (rabbit anti-mouse IgG, HRP conjugated) antibodies after washing 5 times in each step. Finally, the blots were exposed to HRP substrate solution (TMB and H_2O_2) for detection of target protein bands. The densities of ICAM-1 and VCAM-1 bands were determined by Lab works version 4.0 software (UVP, Upland, USA), and the ratios of FAs/control multiplied by 100 and defined as relative expression of ICAM-1 and VCAM-1.

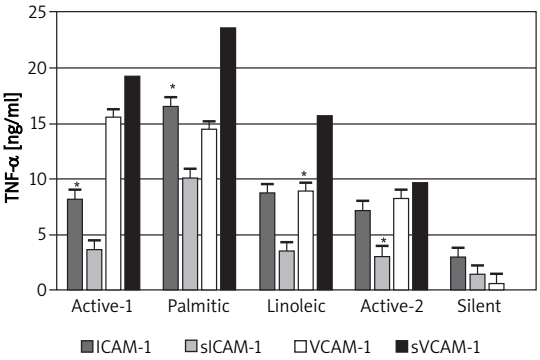

Figure 1. Effect of palmitic and linoleic acid on level of soluble and cell associated ICAM-1 and VCAM-1 in TNF-α -stimulated HBMECs. Cells pre-treated with TNF-α, thereafter with palmitic and linoleic acid (100 μM for 24 h). Active-1 is HBMECs treated with TNF-α (1 ng/ml for 12 h). Active-2 is active-1 cells in culture medium without TNF-α for additional 24 h. Silent is HBMECs in culture medium without TNF-α and fatty acid. Each column shows the mean ± SD of triplicate determinations
**p < 0.05*

Statistical analysis

All tests were performed at least three times. Findings of ELISA considered when variations in intra- and inter-assays were less than 5% and 15%, respectively. The results are expressed as mean ± SD and were analyzed using either Student's *t* test for comparison between two groups or ANOVA (analysis of variance) for multiple comparisons. Results were considered significant when $p < 0.05$.

Results

Cultured HBMECs in the absence of stimulants (LPS or TNF-α) or FAs (palmitic or linoleic acid) expressed very low levels of ICAM-1 or VCAM-1. The results indicated that TNF-α at 1 ng/ml for 12 h and LPS at 10 μg/ml for 12 h induced optimal expression of the adhesion molecules on HBMECs with minimal toxic effects as compared to the control wells. Furthermore, palmitic and linoleic acid up to 100 μM for 24 h or 48 h did not have a considerable adverse effect on cell count and morphology, and protein pattern as assessed by SDS-PAGE (data not shown).

The results of ELISA for assay of cell-associated ICAM-1 showed that TNF-α is a stronger stimulant than LPS for expression of this adhesion molecule. Conversely, LPS could induce sICAM-1 relatively better than TNF-α. Furthermore, LPS could stimulate cell associated and sVCAM-1 very strongly (Figures 1, 2). Therefore, depending on the type of inducer (LPS or TNF-α), one form of the adhesion molecule (soluble

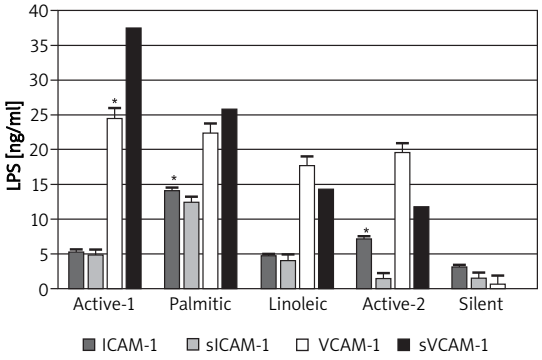

Figure 2. Effect of palmitic and linoleic acid on level of soluble and cell associated ICAM-1 and VCAM-1 in LPS-stimulated HBMECs. Cells pre-treated with LPS, thereafter with palmitic and linoleic acid (100 μM for 24 h). Active-1 is HBMECs treated with LPS (10 μg/ml for 12 h). Active-2 is active-1 cells in culture medium without LPS for additional 24 h. Silent is HBMECs in culture medium without LPS and fatty acid. Each column shows the mean ± SD of triplicate determinations
**p < 0.05*

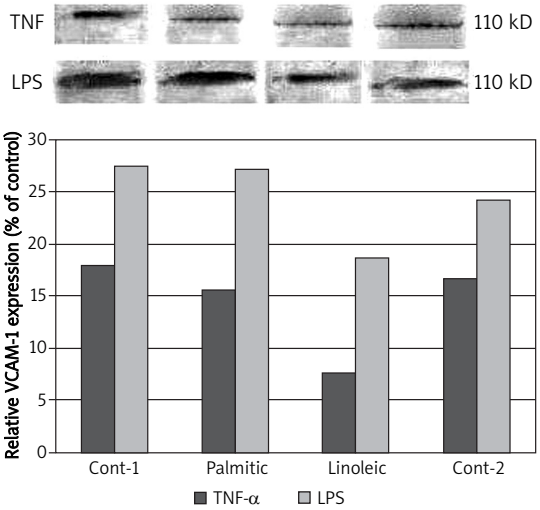

Figure 3. Western blot analysis of VCAM-1 after treatment of HBMECs with palmitic and linoleic acid. Cells stimulated with TNF-α and LPS, and then with palmitic and linoleic acid. Cont-1 is HBMECs grown in medium containing TNF-α or LPS. Cont-2 is cont-1 cells in culture medium without TNF-α or LPS for additional 24 h. Data are representative of three independent experiments

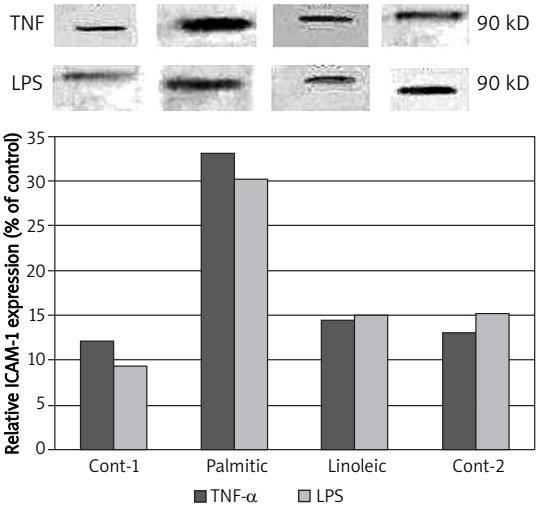

Figure 4. Western blot analysis of ICAM-1 after treatment of HBMECs with palmitic and linoleic acid. Cells stimulated with TNF-α and LPS, and then with palmitic and linoleic acid. Cont-1 is HBMECs grown in medium containing TNF-α or LPS. Cont-2 is cont-1 cells in culture medium without TNF-α or LPS for additional 24 h. Data are representative of three independent experiments

or cell-associated) was up-regulated more than the other form. The results also indicated that these inducers up-regulate VCAM-1 better than ICAM-1, in whole.

When HBMECs induced by LPS (10 μg/ml for 12 h) or TNF-α (1 ng/ml for 12 h) were treated with palmitic or linoleic acid (100 μM for 24 h), the level of the adhesion molecules changed remarkably. Palmitic acid significantly enhanced the level of soluble and cell-associated ICAM-1 (ICAM-1 and sICAM-1) and sustained the level of VCAM-1 and sVCAM-1 in HBMECs when these cells induced with LPS or TNF-α. In contrast, linoleic acid did not significantly affect the level of soluble or cell associated forms of ICAM-1 in HBMECs pre-treated with TNF-α or LPS, but to a small extent enhanced the expression of VCAM-1, especially sVCAM-1, when the cells were activated with TNF-α (Figures 1, 2).

Western blot analysis of total cell-associated VCAM-1 and ICAM-1 following treatment of activated HBMECs with palmitic and linoleic acid is demonstrated in Figures 3 and 4, respectively. These results also indicated that palmitic acid significantly induces the expression of ICAM-1 on HBMECs when these cells are primed with TNF-α or LPS, and sustains up-regulated VCAM-1 at an activated level. In contrast, linoleic acid did not affect the level of ICAM-1 when HBMECs were pre-treated with TNF-α or LPS and VCAM-1 when these cells were pre-treated with TNF-α. However, linoleic acid decreased the level of VCAM-1 when HBMECs were pre-treated with LPS.

Discussion

A number of risk factors for CVD have been identified from epidemiological studies. These include positive family history of CVD, cigarette smoking, hypertension, elevated serum cholesterol, obesity, diabetes, physical inactivity, sex, age and excessive stress [25]. During the 1950s, considerable interest began to develop concerning a possible relationship between dietary fat and the incidence of coronary heart disease (CHD) [26]. The relationship

between saturated fat intake and low density lipoprotein (LDL) is direct and progressive, increasing the risk of CVD [27]. According to the results reported by Bahrami *et al*. [2], edible oil products manufactured in Iran contain remarkably high levels of trans and saturated fatty acids. In addition, the main unsaturated fatty acid in many edible oils consumed in Iran is linoleic acid.

Saturated fatty acids (SFAs) are believed to contribute to induction of CHD [28]. This understanding is based on the evidence that accumulation of SFA in vascular cells leads to lipid-mediated vascular cell dysfunction [29, 30]. Vascular endothelium, as it is continuously exposed to free fatty acids, plays a critical role in the development of vascular-related diseases, including atherosclerosis, CAD, and diabetes [31, 32]. The HBMECs express the cellular adhesion molecules ICAM-1 and PECAM and also VCAM-1 and ELAM-1 upon activation by stimulatory cytokines such as TNF-α [33]. Our results indicated that LPS, in comparison to TNF-α, is a more potent inducer for the soluble form of ICAM-1 (sICAM-1) as well as soluble and cell associated forms of VCAM-1.

Epidemiological studies have shown that soluble ICAM-1 (sICAM-1) is a predictor of CVD among healthy individuals [34-36]. In contrast, levels of soluble VCAM (sVCAM) do not predict primary onset of CVD [36-38] but are markedly elevated in acute coronary syndromes and may be a predictor of death in patients with existing coronary artery disease [39, 40]. It has been suggested that sICAM-1 is a general marker of proinflammatory status in a healthy population, whereas sVCAM-1 is expressed primarily at an advanced stage of atherosclerosis [40]. After up-regulation of the adhesion molecules in HBMECs and treatment of these cells with palmitic or linoleic acid, we found that palmitic acid significantly increases the level of ICAM-1 and sICAM-1, and sustains the level of VCAM-1 and sVCAM-1. The results also indicated that linoleic acid maintains the level of ICAM-1 and VCAM-1 in stimulated HBMECs as assessed by ELISA. However, the western blot results indicated a small decrease in the level of VCAM-1. Therefore, it is suggested that palmitic acid could keep the HBMECs at the stimulated phenotype through increased levels of the two forms of ICAM-1 and maintained level of VCAM-1. These findings are consistent with other studies concerning the effect of palmitic acid on up-regulation of adhesion molecules in endothelial cells such as human coronary artery endothelial cells and human umbilical vein endothelial cells (HUVECs) [5, 41, 42]. Our results also indicated that linoleic acid maintains the level of ICAM-1 at an activated level, and could not lower the VCAM-1 significantly in TNF-α or LPS-induced HBMECs. These findings collectively suggested that the in vitro atherogenic effect of linoleic acid on HBMECs exceeds its anti-atherogenic effect. Our results are in concordance with several reports about the effect of linoleic acid on up-regulation of adhesion molecules and adhesiveness of endothelial cells [43-46]. Both ICAM-1 and VCAM-1 together mediate adhesion of leukocytes to the activated endothelium and transmigration into the sub-endothelial space [47]. Expression of both VCAM-1 and ICAM-1 is up-regulated in atherosclerotic lesions, although VCAM-1 plays a dominant role in the initiation of atherosclerosis [48]. Since the expression of VCAM-1 occurs only on activated endothelial cells, it may be used as a specific marker of plaque burden or activity. Furthermore, levels of VCAM-1 are prognostically important in acute coronary syndromes [49-51]. Most studies report high plasma concentrations of sVCAM-1 and sICAM-1 in coronary artery disease irrespective of stability [52-55].

Collectively, our results indicated that palmitic acid could up-regulate the expression of ICAM-1 and VCAM-1 in HBMECs pretreated with stimulatory agents such as pro-inflammatory cytokines or bacterial LPS. In addition, the results suggested that linoleic acid can sustain up-regulated ICAM-1 and VCAM-1 in activated endothelial cells. Therefore, chronic activation of endothelial cells in the presence of deleterious fatty acids may play a pivotal role in pathogenesis of cardiovascular events. These findings provided further support on the detrimental effects of these fatty acids, specially palmitic acid, in promotion and induction of cardiovascular diseases which are prevalent in the Iranian population. However, more research is needed to characterize the mechanism underlying these effects.

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