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The Effect of Cis and Trans Vaccenic Acids on Expression of ICAM-1 And VCAM-1 in Human Microvascular Endothelial Cells (HMEC)

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A B S T R A C T

Trans-fatty acids (TFA) are known as a risk factor for coronary artery diseases. Trans-11 vaccenic acid [VA; 18:1(n-9)], known as the major trans fatty acid in milk fat, is a positional and geometric isomer of oleic acid and a precursor of conjugated linoleic acid (CLA) in human . This study was carried out in order to investigate the effect of Cis and Trans Vaccenic Acids on expression of intra cellular adhesion molecul-1 (ICAM-1) and vascular cellular adhesion molecul-1 (VCAM-1) in human Microvascular endothelial cells (HMECs). To induce adhesion molecules expression, TNF-α or LPS were added to HMECs in a cell culture being treated with Cis and Trans vaccenic acid. In the next step, to evaluate ICAM-1 and VCAM-1 expression before and after the treatment, Western blot technique was used. The analyzed results indicated that Cis and Trans vaccenic acids are able to suppress VCAM-1 and ICAM-1 expression on HMEC near to the basal level. Also it has been found that cis vaccenic acid isomer could suppress both VCAM-1 and ICAM-1 expression as well as trans isomer. Together all and considering the role of mentioned adhesion molecules in atherosclerosis; it seems that Cis and trans vaccenic acids could be a possible prophylaxis agents in populations with high risk of atherosclerosis.

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Introduction

Adhesion of circulating leukocytes to vessel endothelium is a critical early step in atherogenesis [1]. This process could cause vascular inflammatory reaction as a primary event in the pathogenesis of this vascular disease [2-3]. So far, more than 50 cell adhesion molecules (CAMs) have been identified including immunoglobulin (Ig)-like CAMs, cadherins, selectins, and integrins. Calcium dependent Ig superfamily is the largest family of CAM [4]. Among these type, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial cell E-selectin (E-selectin) are the most prominent adhesion molecules involved in atherogenesis [5-6].However, VCAM-1 have been reported and each of the isoforms has molecular mass (Mr) and Isoelectric point (pI) that could be the result of differential glycosylation states [7].

Tumor necrosis factor-alpha (TNF-α) plays a pivotal role in regulating vascular function in various pathological statuses. It has been shown that this key inflammatory cytokine is a stimulator agent for expression of VCAM-1 and ICAM-1 when an inflammatory condition exists [8]. TNF-α activated human umbilical vein endothelial cells (HUVECs), show an increased adhesion tendency toward leukocytes through over expression of adhesion molecules such as ICAM-1 and VCAM-1 [9].

The relationship between fatty acids and atherosclerosis or other similar inflammatory diseases has been explained by clinical, epidemiological, and in-/ex-vivo studies. An increased intake of saturated fatty acids is positively associated with the development of atherosclerosis and inflammation. In contrast, omega-3 (ω-3) fatty acids, such as docosahexaenoic acid (DHA, C22:6), have proved protective effects against cardiovascular diseases (CVD) [10]. Protective effects of conjugate linoleic acid (CLA) isomers has also been confirmed in-/ex-vivo studies on atherogenesis [11-12], inflammatory bowel disease [13] and oxidative stress [14].

Trans fatty acids (TFAs) are produced via hydrogenation of vegetable oils such as elaidic acid (C18:1 trans-9) and linolelaidic acid (C18:2 trans-9 trans-12) [15]. There's a double bond between cis configurations in mammal's endogenously synthesized unsaturated fatty acids. The main exceptions to mentioned rule are ruminant animals such as cow, sheep and goat, which small amounts of TFA are produced enzymatically through bacterial reactions in their digestion system. (Figure 1) [16]. Trans-11 vaccenic acid [VA; 18:1(n-9)] is the major precursor of CLA in milk fat [17]. Desaturation of VA to rumenic acid (9-cis, 11-trans octadecenoic acid; RA) in tissues is catalyzed by Delta9-desaturase (EC 1.14.99.5) [18]. VA is a positional and geometric isomer of oleic acid and a precursor for conjugated linoleic acid (CLA) in humans. Trans vaccenic acid is a major trans fatty acid in milk fat. Unlike industrially produced trans FAs (mainly elaidic acid), VAs are not considered as a risk factor for CVD. However, this result was in contrast with any study done in an elderly population [17].

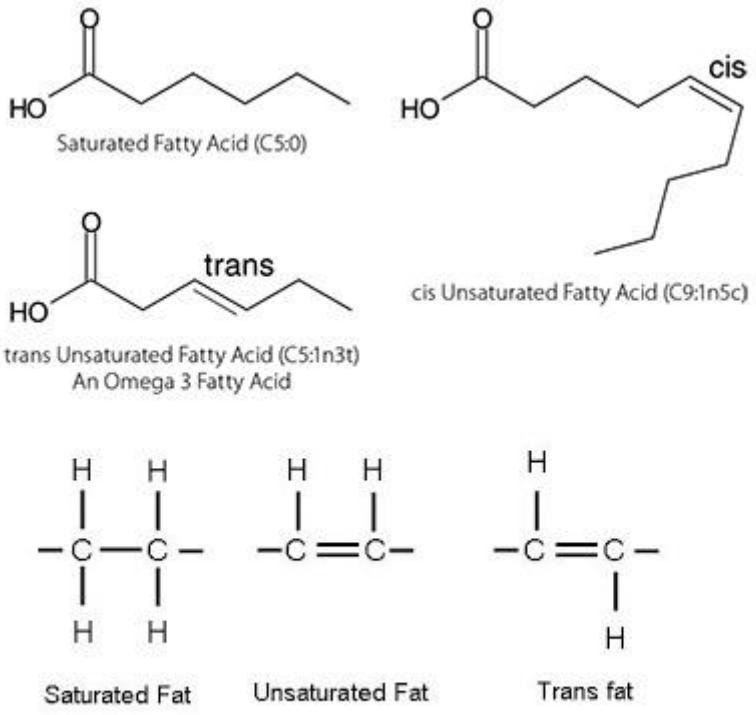

Fig.1. Fatty Acids Structure.

Our previous findings indicated that elaidic acid was able to maintain the level of up-regulated VCAM-1 and ICAM-1 by TNF-α or LPS [5]. Recent studies reported that cis-9, trans-11 CLA isomer reduced VCAM-1 and ICAM-1 expression on the human umbilical vein endothelial cells (HUVECs) [18-19].

Unfortunately, the knowledge of VA bioactivity potential on inflammation, chronic metabolic disorders, including obesity, insulin resistance, and/or dyslipidemia [20] seems not be enough.

The aim of this study was to investigate the possible effects of trans and cis vaccenic acids on expression of VCAM-1 and ICAM-1 on human microvascular endothelial cells (HMECs).

Materials and Methods

Endothelial Cells and Reagents

HMECs were a kindly gift from Dr.Manochehr Mirshahi (College of Medical Sciences, Tarbiat Modares University, and Tehran, Iran). Cells was cultured in enriched DMEM medium containing 25 mmol HEPES, 100 U/L heparin, 2 mmol/L L-glutamine, 1 mol/L sodium pyruvate, 100 U/L penicillin, 100 mg/L streptomycin, (GibcoBRL, Grand Island, NY), bovine serum albumin, free fatty acid bovine serum albomin (FFA BSA). trypsin/EDTA solution, human recombinant TNF-α (PR-430) were purchased from Jena Bioscience, *Escherichia coli* lipopolysacharide (LPS) were purchased from sigma, Monoclonal antibodies (mAb) against VCAM-1 (MAB809), Monocolonal antibodies (mAb) against ICAM-1 (BBA3) were purchased from R&D. Rabbit anti-mouse IgG (HRP Conjugated) prepared in our laboratory.

Cell Culture

HMECs were transferred to serum-free medium for 10 to 12 hours before stimulation by TNF-α or LPS. HMECs were used at 20–25th passages and cultured in a complete medium containing DMEM medium supplemented with 10% FCS, 100 U/L penicillin, 100 µg/mL gentamycin and 10% FFA

BSA at 37 °C and 5% CO2 in a humidified cell culture incubator in 75-cm2 flasks. After reaching confluence, the endothelial cells were treated with trypsin-EDTA and plated to yield near-confluent cultures at the end of the experiment. Cells plated fresh were then allowed to attach in growth medium for at last 48 h. HMECs were pre-treated with TNF-α or LPS to induce adhesion molecules expression. Then they were treated with fatty acids: Trans and Cis VA as would be described in following.

Cytotoxicity assay

The cells were cultured and treated with different concentrations of FAs, TNF-α or LPS. After each incubation period, cell viability was determined by trypan blue exclusion assay. The percentage of cells excluding trypan blue was taken as a measure of cell viability.

Fatty Acid preparation and Treatment

Treatment of HMECs with fatty acids was performed as follows. FA stocks were prepared by dissolving them in 100% ethanol in a final concentration of 5 mg/ml. The prepared stock solution was diluted in serum media to reach FA concentration of 1 mg/ml with corresponding FFA BSA (acting as a FA carrier). The final concentration of ethanol in the media cell culture was calculated 0.1%. Thus, equivalent amounts of BSA and ethanol were added to control plates. The fatty acid/albumin at molar ratios of at 5:1 [21] was added to control samples. The control cells were incubated with DMEM supplemented with 10% FCS in absence of fatty acids, TNF-α, and LPS.

Experimental design

Cultured HMECs was incubated with two different concentrations of TNF-α (1 and 10 ng/ml) and three different concentrations of LPS (0.1, 1 and 10 μg/ml) for 6, 12 and 18 h. This step was taken in order to find the optimal concentration and incubation time for these stimulators to detect the appropriate induction levels of ICAM-1 and VCAM-1. In separate sets of experiments, the cultured HMECs were also incubated with three different concentrations (10, 50 and 100 μM) of FAs (trans and cis vaccenic acid) for 12, 18 and 24h to find out the minimum cytotoxicity dosage. Cell viability (morphology, number and trypan blue exclusion) were assessed. The best concentrations and incubation time which had the maximum viability and protein expression under effect of TNF-α or LPS were evaluated and were selected for further actions. All experiment was performed at least in triplicate.

SDS-PAGE and Western Blot Analysis

Cell lysis was performed using lysis buffer (20 mM tris–HCl pH 7.4, containing 50 mM NaCl, 1% NP-40, 10% glycerol, 1 mM PMSF, 2 mM EDTA). Freshly added protease inhibitor cocktail (Sigma). After 10 minutes keeping in 0° C, the suspension was centrifuged in 14,000×g for 15 min at 4 °C and supernatant was collected. Then the protein extracts were quantified using Bradford assay and equal amounts of total protein (20 μg) were boiled in SDS-PAGE sample buffer for 10 mins and loaded per lane and segregated by 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel at 150 V. In the next step, protein bands were transferred to polyvinylidene difluoride (PVDF). The membranes were washed 3 times with phosphate buffered saline (PBS) containing 0.05% (v/v) tween 20, and then blocked in PBS-BSA 2% (w/v) for two hours. Then it was incubated overnight at 4°C in presence of primary antibodies against VCAM-1, ICAM-1 and 2 hours for secondary (rabbit anti-mouse IgG, horseradish peroxidase conjugated) antibodies after washing 5 times in each step. Finally, the blots were exposed to HRP substrate solution (TMB and H2O2) to detect target proteins. All Western blot analyses were repeated at least three times on different cell culture samples. Blots were quantified by scanning densitometry using Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

Statistical analysis

Data were intended as means ± SEM and evaluated by t-test, one away or two away analysis of variance (ANOVA) appropriate followed by

Tukey test (Graph Pad Software , version 5.0, Inc., La Jolla, CA, USA). A P-value < 0.05 was considered to be statistically significant.

Results

The effects of LPS and TNF-α on Expression of VCAM-1 and ICAM-1 in HMECs

In this study TNF-α and LPS were selected to induce adhesion moleculs due to the effect of pro-inflammatory factors on expression of VCAM-1 and ICAM-1. According to the results, HMECs with no mentioned inflammatory factors treatment, expressed VCAM-1 and ICAM-1 at low basal levels. Effect of pro-inflammatory cytokines on the expression of VCAM-1 and ICAM-1 was tested on the HMECs and results showed that LPS and TNF-α are able to induce VCAM-1 and ICAM-1 as it was predicted before.

The effects of TNF-α, Cis and Trans vaccenic acid on Expression of VCAM-1 and ICAM-1 in HMECs

According to the Western blot analysis results (Figure.2 and Figure.3)HMECs treated with 10 ng/ml of TNF-α significantly induced VCAM-1 and ICAM-1 expression in comparison to 1 ng/ml or non-treated cells. The results showed that VCAM-1 expression in treated cells only with cis and trans VA did not showed a significant difference with controls. Also the expression of TNF-α induced ICAM-1 in HMECs was reduced by cis and VA . Moreover, cis and trans VAs reduced VCAM-1 and ICAM-1 expression weakly when cells were pretreated with TNF-α.

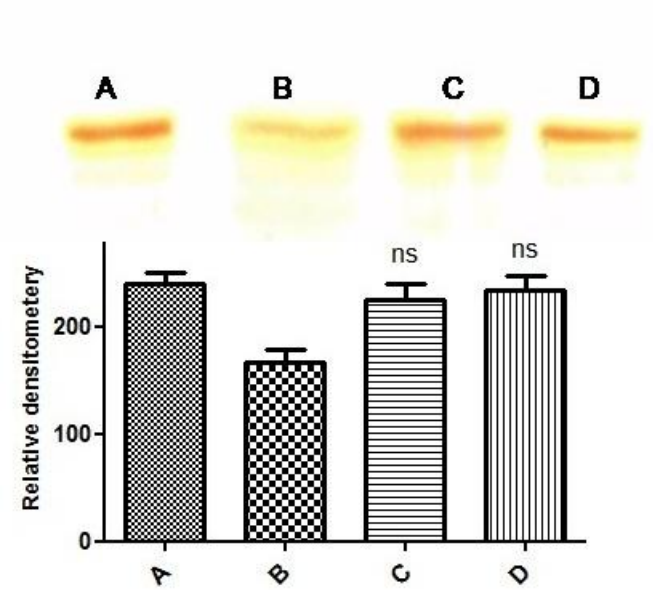

Fig. 2. Western blot analysis of VCAM-1 expression by fatty acids in HMECs stimulated by TNF-α. Cells were pretreated with 10 ng/ml of TNF-α for 12h.(A), Cells were pretreated with TNF-α (B), control (cells incubated without any stimulant and fatty acid) (C), treated with 50μg/ml of cis vaccenic acid for 18h (D), and then treated with 50μg/ml trans vaccenic acid for 18h. Experiments were repeated three times, and the blots are shown as a representative of one of the experiments.

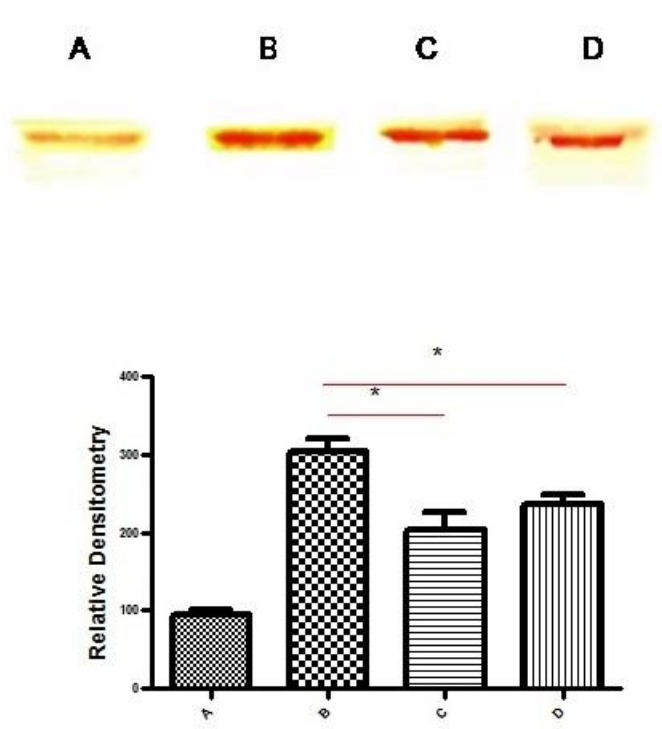

Fig. 3. Western blot analysis of ICAM-1 expression by trans and cis vaccenic acids in HMECs stimulated by TNF-α. Cells were pretreated with 10 µg/ml of TNF-α for 12h. (A), control (cells incubated without any stimulant and fatty acid). (B), Cells were pretreated with TNF-α. (C), then treated with 50µg/ml of cis vaccenic acid for 18h (D), and then treated with 50µg/ml trans vaccenic acid for 18h. Experiments were repeated three times, and the blots are shown a representative of one of the experiments.

The effects of LPS, Cis and Trans vaccenic acid on Expression VCAM-1 and ICAM-1 in HMECs

Western blot analysis indicated that VCAM-1 and ICAM-1 expression after treatment with 10 µg/ml of LPS for 12 h touched an enhancement when compared to unstimulated cells (Figure.4). Also it was shown that cis and trans VAs were able to reduce VCAM-1 and ICAM-1 expression. In addition, western blot analysis indicated that a~60 KDa VCAM-1 isoform expression could be detected in the presence of LPS but not TNF-α. The results showed that reduced VCAM-1 and ICAM-1 expression by cis vaccenic is similar to trans VA (Figure.5).

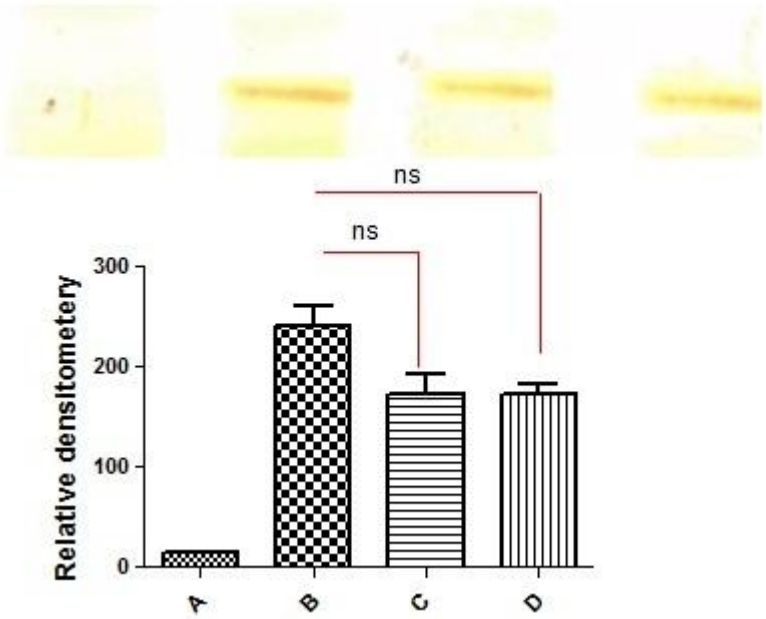

Fig. 4. Western blot analysis of VCAM-1 expression by trans and cis vaccenic acids in HMECs stimulated by LPS. Cells were pretreated with 20 µg/ml of LPS for 12h. (A), control (cells incubated without any stimulant and fatty acid). (B), Cells were pretreated with LPS. (C), treated with 50µg/ml of cis vaccenic acid for 18h (D), and then treated with 50µg/ml trans vaccenic acid for 18h. Experiments were repeated three times, and the blots are shown a representative of one of the experiments.

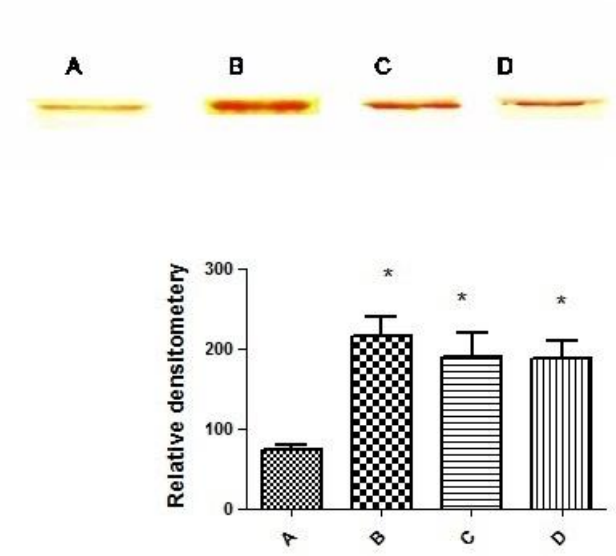

Fig. 5. Western blot analysis of ICAM-1 expression by fatty acids in HMECs stimulated by LPS. Cells were pretreated with 20 µg/ml of LPS for 12h. (A), control (cells incubated without any stimulant and fatty acid). (B), Cells were pretreated with LPS. (C), treated with 50µg/ml of cis vaccenic acid for 18h (D), and then treated with 50µg/ml trans vaccenic acid for 18h. Experiments were repeated three times, and the blots are shown a representative of one of the experiments.

Discussion

Atherosclerosis is one of life threatening vascular disorders which could be discussed from different aspects. One very important phase of these phenomena is inflammation which could be affected by different factors such as ICAMs and VCAMs [5-6]. In this study, the effect of cis and trans VAs on expression of VCAM-1 and ICAM-1 was evaluated on the
HMECs. According to the results, a positive relation between treated cells with trans and cis VA and reduced VCAM-1 and ICAM-1 expression was observed.

Available evidences indicated that trans-18:1 and particular trans-18:2 isomers have stronger effect on cardiovascular heart disease (CHD) than trans-16:1 isomer [22]. Several epidemiological and interventional investigations have revealed a significant association between coronary heart disease and the consumption of TFA [23]. TFA intake has been associated with atherogenic risk factors, such as elevated plasma cholesterol, triglyceride levels and plasma markers of endothelial dysfunction and inflammation [24]. Also industrially produced TFAs have proved to increase serum low density lipoprotein (LDL) cholesterol, decrease high density lipoprotein (HDL) cholesterol [25-26] and increase the risk of CHD [27]. It has been emerged that TFA consumption is associated with significantly higher levels of soluble TNF-α receptors (circulating biomarkers of TNF-a system activity) [28]. Also these fatty acids are able to induce apoptosis in human endothelial cells in vitro [29]. Moreover, TFA intake showed to be associated with increased eTNF-α among healthy women [30] and interleukin-6 (IL-6) and C-reactive protein (CRP) among overweighted women [30]. Our previous study indicated that oleic acid could reduce VCAM-1 and ICAM-1 expression on HMEC, even at the physiological basal amount although elaidic acid is able to increase the level of VCAM-1 and ICAM-1 in TNF-α or LPS pre-treated cells. An in vitro study has demonstrated that TFAs induce pro-inflammatory responses and endothelial cell dysfunction. It has also suggested that TFA intake is associated with higher levels of circulating markers of endothelial dysfunction such as ICAM-1, VCAM-1 and E-selectin [24]. The most predominant trans-isomer in ruminant's TFA is vaccenic acid formed by smaller amounts of conjugated linolenic acid (CLA) [31]. Unlike the industrially produced TFAs (mainly elaidic acid), vaccenic acid seems not to be associated with CHD [15]. However, previous studies do not support the association between ruminant's fat intakes and arthrosclerosis [28]. The aim of present study was to evaluate the hypothesis that trans VA may reduce TNF-α- induced VCAM-1 and ICAM-1expression on HMECs.

Our results clearly indicated that LPS and TNF-α induce the expression of VCAM-1 and ICAM-1. LPS exerts its effects by potently activating macrophages and endothelial cells, and inducing the expression of inflammatory cytokines such as TNF-α and IL-6 [24]. The results of this study showed that cis VA isomer could suppress VCAM-1 and ICAM-1 expression. Also, trans isomer VA reduced VCAM-1 and ICAM-1 expression on the HMECs. Besides, trans VA stimulated HMECs downregulated ICAM-1 and VCAM-1 expression.

The findings clarified that trans VA is not able to induce expression of ICAM-1 and VCAM-1 on HMECs. Following, the results obtained from treatment of HMECs with trans VA after pretreating cells with LPS or TNF-α, exhibited a suppression in ICAM-1 expression on HMEC near the base line levels. Cis-9, trans-11 CLA (Rumenic acid) can be integrated into tissues or biohydrogenated forming trans-11 octadecanoic acid (Vaccenic acid). Therefore it seems that trans vaccenic acid anti-inflammatory specific were displayed and trans vaccenic acid decreases ICAM-1 expression on HMEC. Perhaps trans vaccenic acid anti-inflammatory specifices were displayed when trans vaccenic acid was converted to CLA by Δ9 desaturase. The previous study showed that CLA suppressed the release of cytokines, particularly TNF-a, in animals [32]. On the other hand, another study also reported that cis-9, trans-11 CLA isomer reduced VCAM-1 and ICAM-1 expression on the Human Umbilical vein Endothelial Cell (HUVECs) [18].

The present study may provide a strong evidence for direct effects of trans vaccenic on preventing

arthrosclerosis. These results are consistent with the previous report by (Chantal M. C et al 2010), who demonstrated that trans vaccenic acid protect body against atherosclerosis in animal model.

our previous results obviously revealed that elaidic acid, the most common TFA, sustains the level of ICAM-1 and VCAM-1 expression on HBMEC stimulated by TNF-α or LPS [5].

However, the role of cis and trans vaccenic acids in reducing or inducing inflammation in vivo still requires further examinations. Altogether, this article suggests an immunoblot 2DE gel technique for further, more precise investigations on isoforms 50KDa, 60KDa and 80KDa of VCAM-1 and 70KDa ICAM-1.

Conclusion

Our results have shown that treating HMECs with trans vaccenic acids after pretreating cells with LPS and TNF-α, suppressed ICAM-1 expression on HMEC cell line. Thus it seems to be a possible candidate for the next anti-atherosclerotic studies.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

Acknowledgments

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