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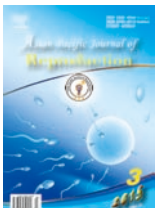

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Lauric acid abolishes interferon-gamma (IFN-γ)-induction of Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) expression in human macrophages

Wei-Siong Lim▽, Mary-Shi-Ying Gan▽, Melissa-Hui-Ling Ong, Choy-Hoong Chew*

Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak, Malaysia

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ABSTRACT

Objective: To investigate the effect of different concentrations of lauric acid on Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) expression in IFN-γ stimulated human monocytic THP-1 cell line. **Methods:** THP-1 cell were cultured using Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum. THP-1 monocytes were firstly differentiated into macrophages by using phorbol-12-myristate-13-acetate. IFN-γ response test was perfomed and total cellular RNA was extracted using TRI Reagent® LS before q-RT-PCR was carried out. Subsequently, IFN-γ treated THP-1 macrophages were stimulated with increasing doses of lauric acid for another 24 hour, before q-RT-PCR. MTT assay was carried out to investigate the effect of lauric acid on undifferentiated and differentiated THP-1 cells. **Results:** The mRNA expression levels of ICAM-1 and VCAM-1 were normalized to β-actin and relatived to the untreated cells. The expressions of ICAM-1 and VCAM-1 were significantly induced in cells treated with 10 ng/mL of IFN-γ. This showed that IFN-γ could up-regulate inflammatory process and may cause atheroma formation. Although lauric acid did not have any significant impact on undifferentiated and differentiated THP-1 cell viability, the normalized fold expressions of ICAM-1 and VCAM-1 in IFN-γ-treated THP-1 macrophages were decreased significantly in a dose dependent manner with the presence of increasing doses of lauric acid. **Conclusions:** This study successfully proved that lauric acid was able to antagonize the up-regulatory effect of IFN-γ on ICAM-1 and VCAM-1 expressions in THP-1 macrophages. This indicates that lauric acid may be an anti-inflammatory therapeutic and prophylaxis agent for atherosclerosis.

1. Introduction

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are classified in Immunoglobulin gene superfamily (IgSF). ICAM-1 is a cell surface glycoprotein receptor that expressed constitutively at basal levels on the surface of different types of cells, which includes endothelial cells, macrophages, fibroblasts, epithelial cells and vascular smooth muscle cells[1]. In contrast, VCAM-1 is expressed on surface of activated endothelium cells, bone marrow fibroblasts, dendritic cells and macrophages[2]. These immunoglobulin supergene family members can be up-regulated in response to the stimulation with phorbol ester or various inflammatory mediators such as immune-regulatory cytokines: Tumor Necrosis Factor-alpha(TNF-alpha) or interferon-gamma (IFN-γ), hormones, virus infection, and cellular stresses[3]. ICAM-1 and VCAM-1 is vastly expressed in atherosclerotic lesions and they function in promoting leukocytes adhesion and development of inflammatory diseases[4]. Thus, ICAM-1 and VCAM-1 could be the potential therapeutic targets for atherosclerosis[5].

IFN-γ is a T-helper cell 1 produced cytokine that serves as a potent activator of macrophages during the innate and acquired immune response[6]. JAK-STAT signaling pathway is activated by the binding of IFN-γ to its receptor. Upon binding, tyrosine kinases are activated and this phosphorylates Signal Transducer

*Corresponding author: Dr. Chew Choy Hoong, PhD, Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak, Malaysia.
▽Both authors have equal contributions to the article.
Tel: + 605-4688888 ext. 4426
Fax: + 605-4661676
E-mail: chewch@utar.edu.my
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and Activator of Transcription-1 (STAT1). Activated STAT1 then dimerizes to regulate the expression of various genes, for instance VCAM-1 and ICAM-1[7, 9–11]. Besides, in various inflammatory and granulomatous conditions, IFN-γ is critical in up-regulating ICAM-1 expression in mRNA levels, amplifying inflammatory cytokine production and enhancing monocytes or macrophages anti-microbial and anti-tumor activity[8].

Lauric acid is a natural saturated fatty acid with 12 carbon atom chain. This medium-chain triglyceride is claimed to have greater anti-viral and anti-bacterial properties[12]. Studies have shown that palm kernel oil, coconut oil and laurel oil are composed of approximately 50 percent of the lauric acid[13]. Lauric acid has been shown to be associated with deleterious effect in atherosclerosis[14]. However, the high content of lauric acid in coconut oil can significantly increase the high-density lipoprotein (HDL) cholesterol level, thus reducing the risk of developing cardiovascular diseases. Therefore, lauric acid is widely used in nutritional and medical applications due to its lack of hypercholesterolemic effects properties[13].

Human monocytic THP-1 cell line is extensively established as a valuable model system for the investigation of macrophages differentiation from monocytes and the regulatory mechanisms of specific genes in macrophages due to the similarities in biological behavior of monocytes or macrophage derived from peripheral blood[15]. Based on these characteristics, the molecular mechanisms regarding the physiological functions of macrophages and monocytes in cardiovascular system as well as the developmental etiology and pathogenesis of the cardiovascular diseases can be widely studied in this cell line[16]. The non-adherent THP-1 cells can be differentiated into adherent macrophages in the stimulation of phorbol-12-myristate-13-acetate (PMA)[8].

Owing to the evidences above, it is hypothesized that lauric acid could relieve the inflammatory effect of IFN-γ and consequently delay or cease the atheroma formation. Therefore, this study was designed to investigate the impact of lauric acid in atheroma formation, particularly investigating the expression of ICAM-1 and VCAM-1 in differentiated THP-1 macrophages, and also to investigate the effect of lauric acid on the cell's viability.

2. Materials and methods

2.1. Cell culture and differentiation

THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Aldrich, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, USA), 2 nM L-glutamine, 10 000 U/mL penicillin and 10 000 μg/mL streptomycin (Milipore, USA) and 2.2 g/L of sodium bicarbonate. The cells were then incubated in a humid incubator with 5% (v/v) CO_2 at 37 ℃ . Cell culture was performed with 75 cm^2 tissue culture flasks (Techno Plastic Products, Switzerland). The cultures were maintained by addition of fresh medium supplemented with 10% (v/v) of FBS or replacement of medium every two or three days of incubation period. Subculture of cells was performed when cell density of 8×10^5 cells/mL was achieved. The cell suspension was then split into (2-4) $\times10^5$ viable cells/mL for each flask.

Prior to cell treatment, differentiation of THP-1 cells was carried out by incubating 1×10^6 cells/mL in 6-well culture plate (Techno Plastic Products) in a final volume of 3 mL of RPMI 1640 medium supplemented with 100 ng/mL of Phorbol Myristate Acetate (PMA) (Sigma Aldrich, USA) and 10% (v/v) of FBS. The six-well plate was incubated for 24 hours at 37 ℃ in a humid incubator with 5% (v/v) CO_2.

2.2. Cell treatment and RNA extraction

The differentiated THP-1 cells were treated with 10 ng/mL of IFN-γ (Milipore) and placed in a humid incubator at a temperature of 37 ℃ with 5% (v/v) CO_2 for 24 hours. Subsequently, the cells were treated with different concentrations of lauric acid (Sigma Aldrich, USA), which were 1 μM, 5 μM, 10 μM, 20 μM, respectively, for another 24 hours. For vehicle control experiment, cells were treated with absolute ethanol (Copens Scientific, Germany). Treatments with 20 μM of lauric acid alone and 10 ng/mL IFN-γ alone for 24 hours, respectively, were used as experimental controls. Total cellular RNA was then isolated from differentiated THP-1 cells using TRI-Reagent® LS (Molecular Research Centre, USA) according to the manufacturer's instructions. Spectrophotometric measurement of total cellular RNA at the ratio of A260/A280 was performed to access the concentration and purity of total cellular RNA. Besides, 1.0% (w/v) agarose-formamide gel electrophoresis was performed to assess the integrity of isolated total cellular RNA.

2.3. Quantitative Reverse Transcription Polymerase Chain Reaction (q-RT-PCR)

Quantitative PCR (qPCR) was conducted by using QuantiTect SYBR Green RT-PCR Kit (QIAGEN) based on the protocol provided by the manufacturer using MyIQ Real-Time PCR Detection System (Bio-Rad). The reactions were assembled on ice to prevent RNA degradation. VCAM-1 qPCR was carried out following a specific protocol, which consists of 1 cycle of 20 minutes at 58 ℃ to synthesis cDNA, followed by 5 minutes at 95 ℃ to inactivate the reverse transcriptase. This was followed by 35 cycles of denaturing at 94 ℃ for 30 seconds, annealing at 63 ℃ for 30 seconds and primer extension at 72 ℃ for 30 seconds. The qPCR protocol for ICAM-1 gene amplification was similar except for the slight change in the annealing temperature to 58 ℃. At the end of each qPCR, melt curve analysis was performed under the following conditions: 1 minute denaturation at 95 ℃, 1 minute annealing at 65 ℃, 81 cycles of 0.5 ℃ increments (10 seconds each) beginning at 65℃ (data collection step). The expression of the target genes was normalized to the housekeeping gene, β-actin, which was used as an internal control to obtain the relative mRNA expression of the VCAM-1 and ICAM-1. Relative quantification of the expression of targeted genes to β-actin was carried out using Pfaffl method[17]. Furthermore, the experiments were carried out in triplicates for each RNA sample to determine the precision of q-RT-PCR results and to minimize technical errors that would cause differences in expression. The primers which were used for q-RT-PCR were taken from Park *et al.*[18].

2.4. Statistical analysis

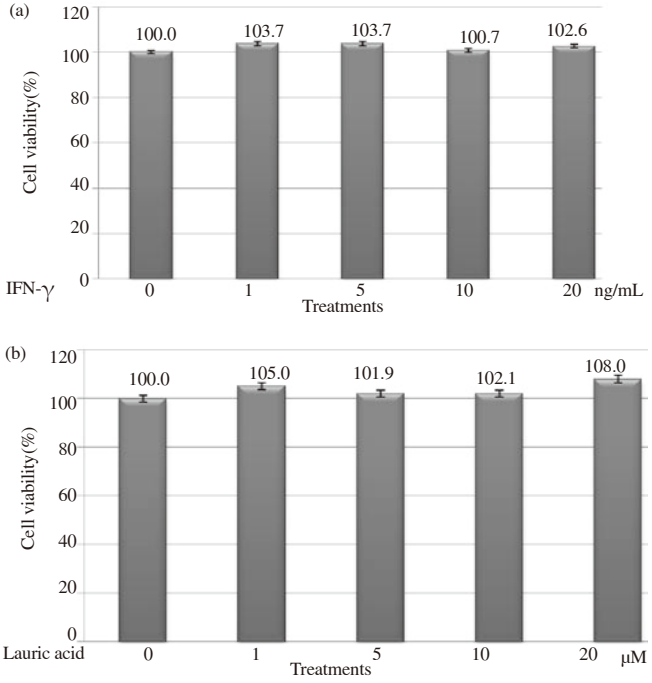
Results were presented as means and standard deviations of triplicate determination. IBM's Statistical Package for the Social Sciences (SPSS) Statistics (IBM Corporation) was used for statistical analysis. Statistical significance was determined using student-paired *t*-test as *P*-value (p) less than 0.05 ($P<0.05$) was considered statistically significant.

3. Results

3.1. Effect of IFN-γ, lauric acid alone or combined treatment on undifferentiated and differentiated THP-1 cell viability

Figure 1 depicts the undifferentiated THP-1 monocytes' viability under effects of different doses of IFN-γ and lauric acid. The cell viability of undifferentiated THP-1 cells remained in a stable pattern when IFN-γ doses were increased. Increasing concentration of lauric acid from 1 μM to 20 μM also did not affect the undifferentiated THP-1 cells' viabilities. Interestingly, the cell viability was decreased insignificantly to 79.3% when the THP-1 cells were treated in both 20 μM of lauric acid and 10 μM of IFN-γ, despite the viability of the cells was fairly stable in the co-stimulation of IFN-γ with other doses of lauric acid.

An insignificant decline in the cell viability of THP-1 macrophages treated with increasing doses of IFN-γ (Figure 2a) and varied concentrations of lauric acid (Figure 2b), in relative to the untreated sample. The cell viability percentage in THP-1 macrophages treated with the same amount of absolute ethanol used to dissolve lauric acid for 20 μM treatment as a control. Although an increasing trend in cell viability was observed in IFN-γ-stimulated THP-1 macrophages with increasing concentrations of lauric acid, statistical analysis showed no significant cell viability change under these treatments (Figure 2c).



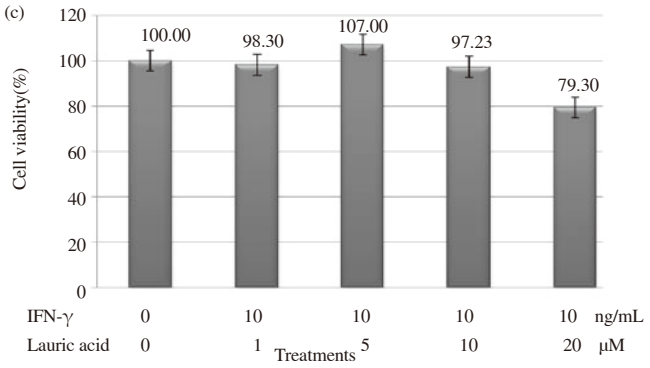


Figure 1. The effect of (a) IFN-γ (b) lauric acid alone and (c) combined 10 ng/mL of IFN-γ and lauric acid on undifferentiated THP-1 cell viability. The X-axis represents the different concentrations of IFN-γ and lauric acid, while the Y-axis represents the percentage of cell viability. The values above each bar signify the percentage of cell viability in each treatment. Standard deviation is also shown as error bar.

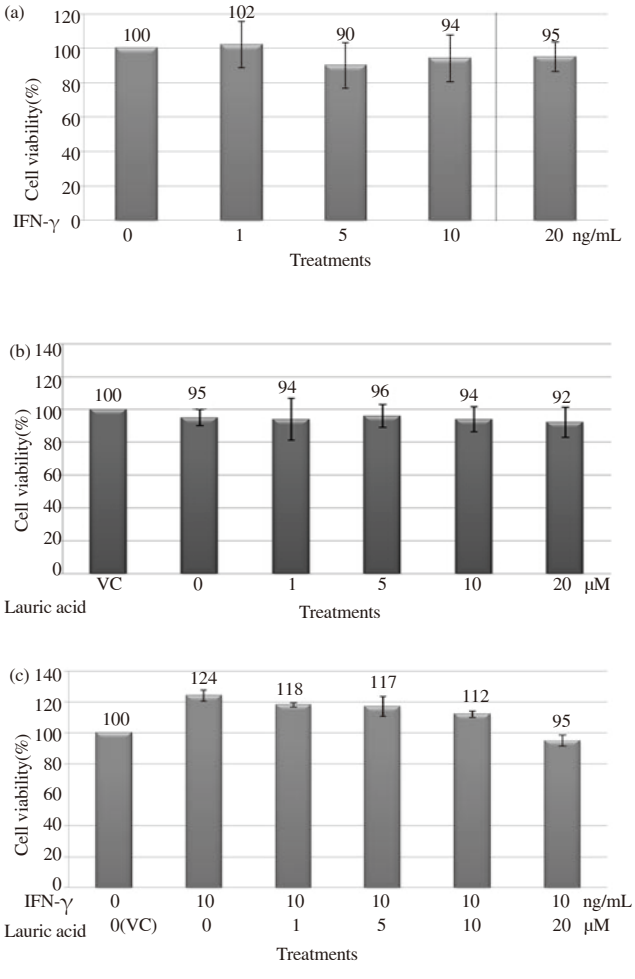


Figure 2. The effect of (a) IFN-γ (b) lauric acid alone, and (c) combined 10 ng/mL of IFN-γ and lauric acid on differentiated THP-1 macrophages cell viability. The X-axis represents the different concentrations of IFN-γ and lauric acid, while the Y-axis represents the percentage of cell viability. The values above each bar signify the percentage of cell viability in each treatment. Standard deviation is also shown as error bar.

3.2. Effect of IFN-γ, lauric acid alone or combined treatment on ICAM-1 and VCAM-1 expression

Figure 3a depicts the expression profile of VCAM-1 after the cells were treated with different doses of lauric acid. IFN-γ induced the expression of VCAM-1 gene to 1.28-fold. The expression of VCAM-1 gene was suppressed to 0.30-fold by 20 μM lauric acid treatment without IFN-γ. Besides, VCAM-1 expression was also suppressed to 0.66-fold, 0.72-fold and 0.67-fold in post-treatment of IFN-γ pre-stimulated cells with 5 μM, 10 μM and 20 μM of lauric acid, respectively.

The normalized fold expression of ICAM-1 under the treatment of 10 ng/mL of IFN-γ (1.32-fold) was used as a control to investigate the effect of post-stimulation with lauric acid on IFN-γ-induced ICAM-1 mRNA expression (Figure 3b). Similar to VCAM-1, ICAM-1 mRNA expression with only 20 μM of lauric acid was down-regulated to 0.63-fold as compared to the untreated sample. Although ICAM-1 expression was induced in the presence of IFN-γ, the induction was decreased to 0.93-fold, 0.55-fold, 0.57-fold and 0.57-fold after the stimulation with 1 μM, 5 μM, 10 μM and 20 μM of lauric acid, respectively. This result shows that lauric acid suppressed the IFN-γ induction of ICAM-1 and VCAM-1 expression in THP-1 macrophages. The degree of reduction in ICAM-1 and VCAM-1 expression remained rather constant after post-treatment with 5 μM of lauric acid, and thus, this indicates the optimal dosage of lauric acid.

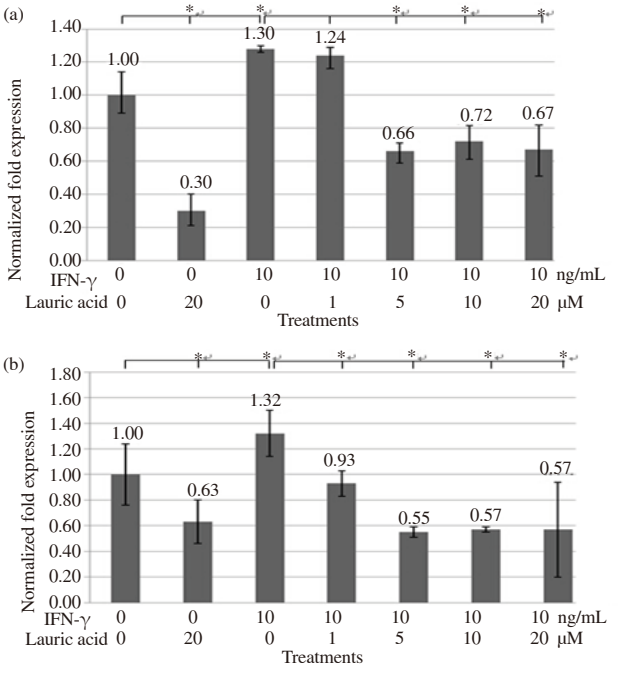


Figure 3. Dose response of (a) VCAM-1 and (b) ICAM-1 expression in THP-1 macrophages treated with 10 ng/mL of IFN-γ and different concentrations of lauric acid.
The X-axis represents the concentrations of lauric acid while the Y-axis represents the normalized fold expression of VCAM-1 and ICAM-1. The values above each bar indicate the fold expression values of VCAM-1 and ICAM-1, which were normalized to β-actin and relative to untreated cells. The error bar represents the standard deviation. The values are expressed as mean ± SD; $n = 3$ (triplicate data) for all experiments. $P< 0.05$ which is represented by * is considered statistically significant change from untreated THP-1 cells or IFN-γ- treated THP-1 cells.

4. Discussion

In this study, IFN-γ up-regulated VCAM-1 and ICAM-1 mRNA expressions were significantly reduced by lauric acid. IFN-γ has been shown to increase the expression of VCAM-1 and ICAM-1. Induction of VCAM-1 gene expression by IFN-γ could happen due to activation of nuclear factor kappa B (NF-κB) and interferon regulatory factor-1 (IRF-1) expression in response to IFN-γ stimulation. Both NF-κB and IRF-1 are transcription factors which bind to VCAM-1 promoter and induce VCAM-1 gene transcription [19].

Lauric acid is a fatty acid with anti-oxidant and anti-inflammatory effects[20]. Lauric acid has been shown to reduce the expression of transcription factor, NF-κB[21]. NF-κB is a pro-inflammatory transcription factor that activates immune and inflammatory response by controlling the genes expression of a wide spectrum of inflammatory cytokines and cell adhesion molecules[22-24]. Hence, the reduction of IFN-γ induced VCAM-1 and ICAM-1 expression might be caused by the diminished cellular NF-κB concentration after the treatment of lauric acid.

Lauric acid has been proven to possess anti-oxidative activity[20]. Hence, lauric acid could scavenge reactive oxygen species (ROS) [29]. ROS is inflammatory mediators which could induce the up-regulation of VCAM-1 expression in endothelial cells during inflammation[20]. Also, free fatty acid was proven to affect the production of mtROS which act as signaling molecules to induce the production of pro-inflammatory cytokines and increase expression of VCAM-1 during cellular injury[25-26]. Therefore, lauric acid is hypothesized to reduce the expression of IFN-γ induced expression of VCAM-1 by removing ROS during inflammation.

Here we show that lauric acid down-regulated the expression of VCAM-1 and ICAM-1. The mechanism involved could be due to its ability to inhibit cyclooxygenase-II (COX-II) isoform expression [27-28]. Cyclooxygenase (COX-I and COX-II) are enzymes which function to convert arachidonic acid to prostaglandins which control the inflammation process in the body. Furthermore, COX-II has been shown to limit the expression of the adhesion molecules in human vascular smooth muscle cells[29]. One possible mechanism is for lauric acid to act through COX-II induction. However, this possibility remains to be examined.

In conclusion, lauric acid was able to alleviate the up-regulatory effect of IFN-γ on ICAM-1 and VCAM-1 expression at transcriptional level in THP-1 macrophages, without affecting the cell viability. This may be important for the control of inflammatory diseases, for instance atherosclerosis. Thus, lauric acid could be a potential therapeutic agent for inflammatory related diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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