Effect of Eicosapentaenoic Acid on the Expression of ABCG1 Gene in the Human Monocyte THP-1 Cells

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Abstract- Cardiovascular disease (CVD) is the leading cause of death and disability in developed countries. Atherosclerosis is the major cause of CVD, accounting for about half of the attributed deaths. Cholesterol homeostasis is one of the most important factors in atherosclerosis. ATP-Binding cassette transporters cholesterol. Omega (ω) 3 fatty acids are important ligands for regulation of ABC transporters such as ABCG1. Concern has been raised that the low absolute intakes of EPA and high ratios of ω-6 polyunsaturated fatty acids (ω-6 PUFA) to EPA may predispose some individuals to CVD. Eicosapentaenoic acid (EPA) is the most abundant ω3 fatty acid in the diet. The objective of this study was to evaluate the effect of different concentrations of EPA on the expression of ABCG1 gene in the human monocyte THP-1 cells. In this study, THP-1 cells were cultured in RPMI 1640 medium, THP-1 monocytes were then differentiated to macrophages with PMA (phorbol myristic acid) and stimulated with 50, 75 and 100 μM of EPA for 24 h at 37°C. We examined the effects of EPA treatment on the expression of ABCG1 gene using Quantitative Real time RT-PCR (qRT-PCR). Our results, indicate that ABCG1 mRNA expression was significantly reduced by 50, 75 and 100 μM EPA fatty acid treatments as compared to the control cells ($p = 0.009$, $p < 0.001$ and $p = 0.002$, respectively). These results suggest that polyunsaturated fatty acids (PUFAs) such as EPA have an effect on the cholesterol homeostasis in macrophages, and they can change the expression of ABCG1 gene. It seems that EPA has different effects on gene expression and lipid metabolism.

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Introduction

Coronary artery disease (CAD) is one of the major causes of mortality in developed countries and multiple factors contribute to the formation of lesions that ultimately lead to CAD (1-4). Atherosclerosis is a disease of arteries and is characterized by endothelial dysfunction, vascular inflammation, and the build-up of lipids, cholesterol, calcium, and cellular debris within the intima of the vessel wall (5). A major event in the progression of atherosclerosis is the differentiation of monocytes to macrophages that accumulate lipoprotein-derived cholesterol to form foam cells within the arterial wall (6,7). Defect in Cholesterol homeostasis is one of the most important factors that can cause initiation and progression of atherosclerosis. Excess unesterified cholesterol (UC) is toxic to cells; therefore, cells have developed pathways to protect themselves against cholesterol toxicity. One key pathway is the efflux of cholesterol to extracellular "acceptors" via cholesterol exporters like ATP-binding membrane cassette transporter A-1 (ABCA1) and ATP-binding membrane cassette transporter G-1 (ABCG1)(8,9).

ATP-binding cassette (ABC) transporters are one of the largest families of integral membrane proteins(10,11) .These proteins transport a wide variety of substrates including amino acids, lipids, lipopolysaccharides, inorganic ions, peptides, sugars, metal ions, drugs and proteins(10-12). ABC transporters utilize the energy derived from ATP binding/ hydrolysis

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to drive substrate translocation across the membrane (13,14).The human ABCG subfamily contains five characterized half-transporters (ABCG1, ABCG2, ABCG4, ABCG4, ABCG5 and ABCG8)(10).

Studies in the past years have demonstrated that ABCG1 (formerly termed ABC8) plays a critical role in regulating cellular cholesterol homeostasis (15).

Long-chain polyunsaturated fatty acids (PUFAs) are good candidates as putative regulators of ABCG1 gene(15).One component of the diet that appears to play a multifactorial role in CVD risk is very long chain omega (ω)-3 fatty acid, eicosapentaenoic acid (EPA, C20:5 ω-3) . Observational data in humans suggest that the weekly consumption of 1 to 2 times of fish per week which contains EPA is associated with decreased CVD mortality(16).The aim of this study was to evaluate the effect of various concentrations of EPA on the expression of ABCG1 gene in THP-1 cell line by real time PCR.

Materials and Methods

Chemicals

RPMI 1640 medium, penicillin, phosphate buffer saline (PBS) and streptomycin were obtained from Invitrogen Corporation. Eicosapentaenoeic acid (purity >99%), phorbol 12-myristate13-acetate (PMA), and dimethyl sulfoxide (DMSO) were purchased from sigma-Aldrich, USA. THP-1 cell lines were obtained from the cell bank of Iran (Pasteur institute, Iran). Serum Free Media (SFM) and fetal bovine serum (FBS) were purchased from Gibco-BRLReagents .All the kits for RNA extraction and reverse transcription were obtained from Qiagen, USA. SYBR Green PCR Master Mix Reagent and qRT- PCR Primers (ABCG1, β-Actin) were purchased from Qiagen, USA. Also, other chemicals were obtained from Sigma.

Cells and Cell Culture

Human THP-1 cells, a human monocyte cell line, were cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin, 50× 10−3 mol/m3 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine and 1.5 g/L sodium bicarbonate at 37°C in 5% CO2 at a cell density of 1.0×106 /mL.

EPA Preparation

A stock of 100 mM EPA in DMSO was prepared.This stock solution was aliquoted and frozen. Fresh fatty acid was prepared before every experiment from the stock solution. EPA was added from the stock solution to 100 volumes of 10% BSA/medium solution and incubated for 2 h at 37° C to form EPA conjugates. Once the EPA was completely dissolved, the media pH was balanced, and filter sterilized through a 0.2-µm syringe filter.

Cell Differentiation and Treatment with EPA

Cells were centrifuged at 200 g for 10 min, the pellet was resuspended in RPMI without fetal calf serum and cell concentration was adjusted to 106 cells/mL. THP-1 cells were washed with PBS. Then the THP-1 cells were treated in a medium containing100 ng/mL PMA for 24 h to differentiate to macrophages. According to study of Ringseis *et al*., THP-1 cells treated with PMA became flat and amoeboid in shape, and adhered to the surface of cell culture plates, that is associated with the macrophage phenotype (Figure 2B)(17) .

Cells were treated with 50, 75 and 100 ×10−3 mol/m3 of EPA for 24 h at 37°C. The control group consisted of PMA-differentiated THP-1 cells treated with DMSO. All experiments were conducted as triplicates.

Cell Proliferation and Cytotoxicity Assay

The viability test was performed. Briefly, cell cytotoxicity assay was assessed by measuring the activity of mitochondrial dehydrogenase. 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) reagent was used. After incubation at 37°C for 1 h, the absorbance at 490 nm was assayed using an ELISA plate reader (Biotech).

Total RNA Isolation and Quantitative Real-time Polymerase Chain Reaction (qPCR)

In order to assess the expression of ABCG1 gene, THP-1 cells were incubated with 50, 75 and 100× 10−3 mol/m3 EPA for 24 h. Total RNA was isolated by RNeasy Mini kit (Qiagen, USA) following manufacturer's protocol. Quality and integrity of the RNA was tested on 1.5% agarose gel electrophoresis and ethidium bromide staining. RNA concentrations were determined spectrophotometrically by absorbance measurement at 260 / 280 nm. Reverse transcription for complementary DNA synthesis and quantitative real-time PCR analysis were performed, as per previous studies (18). ABCG1 mRNA analysis was done on a Corrbet® sequence detection system (Rotor gene 6000) using SYBR Green PCR master Mix (Applied Biosystems) following manufacturer's protocol. Amplification conditions were 5 min at 95°C

(denaturation), 10 s at 95°C (annealing), 30 sec at 60°C (extension) for 40 cycles. Expressions of ABCG1 were normalized against the internal control β-actin gene. Relative quantification for gene expression was calculated using 2-Δ (CTA-CTB) formula.

The sequence of the primers used for RT-PCR analysis was as follows: ABCG1, forward (5′-CGGAGCCCAAGTCGGTGTG-3′), and reverse (5′-TTTCAGATGTCCATTCAGCAGGTC-3′); Beta actin forward (5′-CTGGAACGGTGAAGGTGACA-3′), and reverse (5′-AAGGGACTTCCTGTAACAATGCA-3′).

Statistical Analysis

One - way analysis of variance (ANOVA) was used to identify statistically significant differences of treatments. To analyze EPA effect on ABCG1 mRNA expression, $P < 0.05$ was considered significant, and data expressed as mean ± SD. Statistical analyses were performed using SPSS version 13.0.

Result

Cell Viability by MTT Assay

To investigate whether EPA treatment can cause cell toxicity, the effects of different concentrations of EPA on cell viability were compared using MTT assay, the difference in cell viability between different treatments was not statistically significant. These results indicate that the experimental concentrations of EPA did not induce cell death. As shown in Figure 1 maximum concentration of conjugated eicosapentaenoic acid (EPA) (100 ×10−3 mol/m3) was not toxic for the THP-1 cells. Figure 2 shows macrophages obtained from THP-1 cell differentiation after phorbol myristic acid treatment.

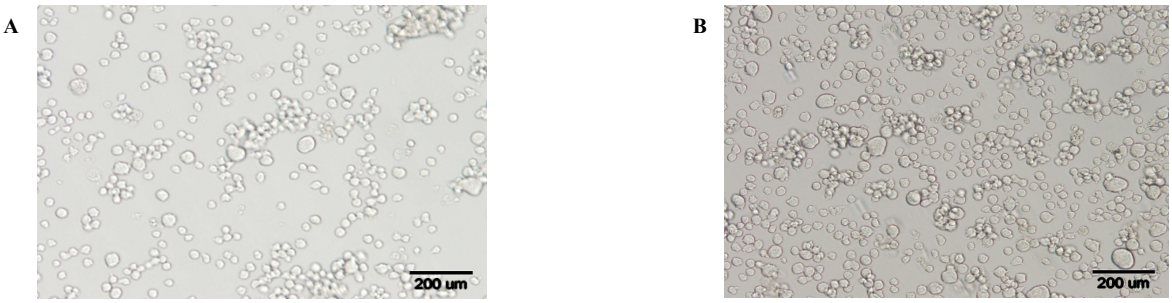

Figure 1. A) Growth of DMSO-treated THP-1 cells. B) Cell viability in the presence of 100 µM of EPA. Normal growth of THP-1 cells in the presence of 100 micromoles of EPA.

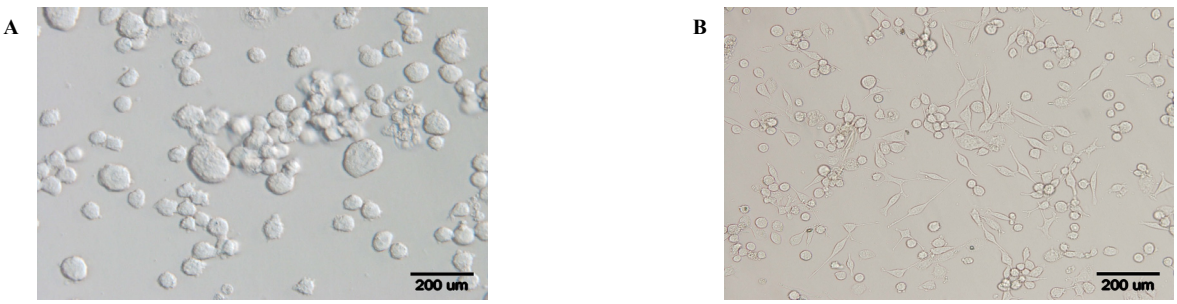

Figure 2. A) Morphology of undifferentiated THP-1 control cells.
B) Differentiation of THP-1 cells to macrophages after phorbol myristate acetate (PMA) treatment for 72 hours.

Total RNA Isolation

For the determination of the integrity of RNA extracted from macrophage cells, agarose gel electrophoresis was performed in the presence of various concentrations of EPA .Figure 3, 18 S rRNA and 28 S rRNA bands indicated RNA purity and integrity was appropriate for the experimental procedure.

Decreased ABCG1 mRNA Expression in Human Macrophages Treated with Different Concentrations of EPA

In order to evaluate the effect of eicosapentaenoic acid on the ABCG1 gene expression in THP-1 cells, treated with various concentrations of this fatty acid, quantitative real-time PCR (qRT-PCR) was performed. Incubation of cells with 50, 75 and 100 (×10−3 mol/m3)

of EPA for 24 h significantly decreased mRNA expression as compared to control group ($p = 0.009$, $p < 0.001$ and $p = 0.002$, respectively) (Figure.4).

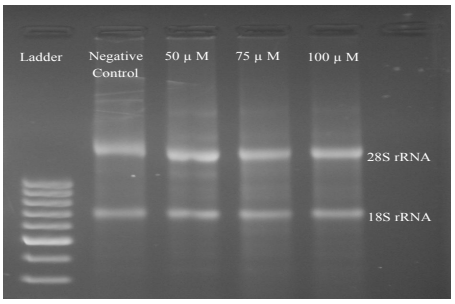

Figure 3. Total RNA extracted from differentiated THP-1 cells in the presence of various concentrations of EPA

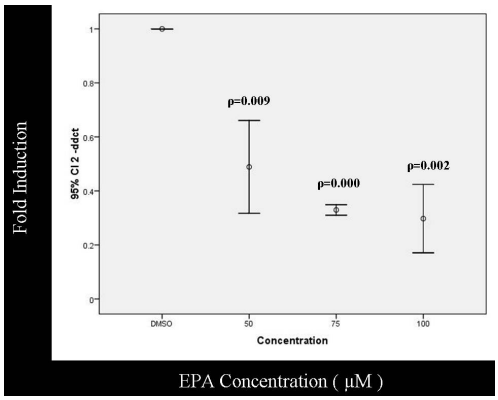

Figure 4. Effects of various concentrations of EPA (50, 75 and 100 µM) on ABCG1 mRNA expression in human THP-1 derived macrophages. Cells were treated with different concentrations of EPA for 24 h. RNA extracts and assayed using quantitative real-time RT-PCR (qRT-PCR) as described above

These results are normalized to an internal control gene (B-actin). All results were tested in triplicate and compared with control (DMSO). Data represent the means ± SD of three independent experiments

Discussion

Atherosclerosis, an inflammatory disorder, is a major phenomenon, which makes individuals susceptible to myocardial infarction. Arising evidence from variety studies suggests that macrophages have a key role in the initiation, progression, and development of atherosclerotic plaques. Also, it seems they are important in plaque rupture. Macrophages undergo activation, migration, differentiation, proliferation, and death during atherosclerosis development and progression .Therefore cellular signaling mechanisms controlling these events are important therapeutic interventions of future research is required in this area before researchers can formulate a common strategy for targeting macrophages in atherosclerosis. Since many data regarding the intracellular mechanisms involving this phenomenon in literature are diverse and upcoming, real challenge lay in connecting them and drawing a meaningful conclusion (19).

Despite increasing interest and extensive study on the role of ABCG1, relatively little is known about how these transporters prompts efflux of cholesterol to mature HDL (20). ABCG1 is a member of the ATP-binding cassette transporter superfamily, which has been mapped to chromosome 21q22.3 (21-26). In contrast to ABCA1, which is a full ABC transporter with two ATP binding cassettes and transmembrane domains, ABCG1 is a half transporter containing only one ATP binding cassette and one transmembrane domain(24,25).It is, therefore, believed to need a dimeric partner for being active. Wang et al. has reported that ABCG1 contributes to HDL2- and HDL3-dependent cellular cholesterol efflux 8.

ABCG1 is one of the key players in reverse cholesterol transport and is critical in regulating cellular cholesterol homeostasis (24,25). To shed light on EPA effect on ABCG1, current study was undertaken to investigate the effect of EPA on the ABCG1 gene expression. As shown in results, EPA decreased ABCG1 expression at the transcriptional levels in a dose-dependent manner after 24 h incubation.

Wang et al recently showed that ABCG1 promotes efflux of cholesterol to a variety of acceptors including HDL, LDL, and phospholipid vesicles without increasing the binding of lipoproteins to cells (20).

In the previous study in this laboratory the role of eicosapentaenoic acid (EPA) in the regulation of the ATP-binding cassette A1 (ABCA1) and liver X receptor α (LXR) genes, which are involved in cholesterol homeostasis has been investigated (18). Results showed that this fatty acid significantly reduced the total, free and esterified cholesterol in the foam cells. While the expression of the ABCA1 and LXRα genes was not significantly affected by EPA. These results suggest that although polyunsaturated fatty acids have an effect on cholesterol homeostasis, they did not change the expression of the ABCA1 and LXRα genes (18).

Although in other studies LXRs have been shown to prevent atherosclerosis progression by positively modulating the expression of genes involved in reverse cholesterol transport like ABCA1, ABCG1, ABCG5, ABCG8, and ApoE (27).

In addition, it has been shown that SREBP-1c can upregulate ABCG1 transcription, and this has been confirmed by microarray and promoter analysis experiments (28).

On the other hand, Peroxisome proliferator-activated receptor (PPAR) forms a heterodimer with RXR and gets activated in the presence of fatty acids (29).

This activated heterodimer binds to the response element of its target gene LXR-a, and upregulate its expression. PPAR may exert their protective effect by upregulating LXR (29).

However, in HepG2 and Caco-2 cells PPARγ activation by troglitazone lowers the cholesterol synthesis by reducing the concentration of nuclear SREBP-2 and successive down regulation of its target genes involved in cholesterol synthesis (30).

These results indicate that PPARγ and SREBP-2 interaction and regulation may be tissue/cell-specific, which makes the targeting of this pathway more challenging.

The success of PPARγ and PPARα ligands will depend on the understanding of their basic structure and function and use of strategies focusing selective modulation of their activities intissue and target gene-specific manner.

This study was designed to assess the effect of various concentrations of EPA on the ABCG1 gene expression in THP1cell line. Results obtained in the present study demonstrated that EPA lowers ABCG1 expression in THP1 cells.The real mechanism of EPA effect on ABCG1 gene expression is rather complicated; therefore further studies are required for better understanding of PUFAs and ABCG1 gene role in cholesterol hemostasis.

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