

Compare the Effect of Eicosapentaenoic Acid and Oxidized Low-Density Lipoprotein on the Expression of CD36 and Peroxisome Proliferator-Activated Receptor Gamma

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ABSTRACT

Background: There is evidence that CD36 promotes foam cell formation through internalizing oxidized LDL (ox-LDL) into macrophages; therefore, it plays a key role in pathogenesis of atherosclerosis. In addition, CD36 expression seems to be mediated by nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR- γ). The aim of the present study was to evaluate and compare the effect of PPAR- γ ligands, eicosapentaenoic acid (EPA) as an anti-atherogenic factor and ox-LDL as an atherogenic factor on CD36 expression. Mechanism of PPAR- γ action and its ligands in CD36 expression were also investigated. **Methods:** Raw 264.7 macrophage cell line was treated with ox-LDL (100 and 150 μ g protein/LDL) and EPA (100 and 200 μ M) for 24 and 48 hours in absence or presence of PPAR- γ inhibitor, T0070907. Quantitative real-time PCR and Western-blotting were used for analysis of gene and protein expression, respectively. **Results:** Raw 264.7 exposures to ox-LDL and EPA resulted in increased expression of CD36 mRNA and protein; however, mRNA and PPAR- γ protein were not up-regulated significantly. Pre-incubation of cells with T0070907 led to decreased expression of CD36 when treated with ox-LDL and EPA. **Conclusion:** It was confirmed that both EPA and ox-LDL increased CD36 expression but not PPAR- γ , and also co-treatment with PPAR- γ inhibitor decreased CD36 expression. We concluded that up-regulation of CD36 depends on PPAR- γ activation and is not related to increased expression of PPAR- γ . Induction of CD36 by EPA showed that CD36 suppression is not the means by which ω -3 fatty acids (EPA) provide protection against formation of atherosclerotic plaque. *Iran. Biomed. J. 17 (2): 84-92, 2013*

Keywords: Atherosclerosis, proliferator-activated receptor gamma (PPAR- γ), Oxidized low density lipoprotein (ox-LDL), Eicosapentaenoic acid (EPA)

INTRODUCTION

The accumulation and retention of LDL, especially its oxidized form (ox-LDL) plays an important role in the formation and development of atherosclerotic plaques [1]. *In vivo*, LDL is oxidized in the sub-intimal of the artery by contacting with reactive oxygen species, resulting in induced oxidation of cholesterol and polyunsaturated fatty acids (PUFA) in LDL [2]. Ox-LDL uptake by macrophage (located at sub-endothelial of arteries) results in the accumulation of cholesterol in these cells. This scenario promotes transformation of macrophages to foam cells and, over time, formation of the atherosclerotic plaques and atherosclerosis [3].

The uptake of ox-LDL is mediated by various scavenger receptors existing on the surface of macrophages. Among them, CD36 (an 88-kDa

glycoprotein) is likely the most important and responsible for 50% macrophage lipid uptake [4, 5]. It has been also shown that CD36/apo E double-null mice have smaller atherosclerotic lesions than apo E-null mice when fed the atherogenic diet [6].

CD36 is expressed by different types of cells, including adipocytes, endothelial cells, macrophages, skeletal muscle cells and platelets [7]. It recognizes numerous ligands, including fatty acid, ox-LDL, thrombospondin and apoptotic cell [7]. It is known that regulation of CD36 is at transcription level, and different studies have revealed various responses in different tissues [8, 9].

The biological effects of ox-LDL are mainly mediated via signaling pathway and activation of transcription factors [10]. Ox-LDL promotes the expression of target genes that are involved in inflammation and stress oxidative condition. Some

published data suggest that, ox-LDL induces the expression of CD36, likely through activation and expression of peroxisome proliferator-activated receptor gamma (PPAR- γ) [11, 12] as some components of ox-LDL function as PPAR- γ ligands [13]. PPAR- γ is a ligand-dependent transcription factor that belongs to the steroid hormone receptor superfamily. It plays a role in some cell-physiological activities, including adipocytes differentiation, glucose homeostasis, inflammatory responses as well as foam cell formation [14]. It has been shown that CD36 promoter has PPAR response element and that ox-LDL increases activity of this promoter through PPAR- γ [15].

It is also known that fatty acids especially PUFA such as eicosapentaenoic acid (EPA) are ligands of PPAR- γ and have an important role in inhibition of inflammation. One recent paper has reported that EPA block the inflammatory effect of TNF- α on muscle cells and this anti-inflammatory potent of EPA is dependent on PPAR- γ [16].

There is increasing evidence that dietary omega-3 fatty acids such as EPA and DHA (docosahexaenoic acid) have a beneficial role in formation of foam cells and atherosclerotic lesions, but the exact signaling pathway has not been clearly understood yet. Therefore, the effect of EPA as an anti-atherogenic factor on the expression of CD36 and PPAR- γ was investigated to discern whether EPA and ox-LDL have the same effect on expression of these two genes.

Although it seems that both ox-LDL and EPA are ligands of PPAR- γ , the role of PPAR- γ in expression of CD36 in response to ox-LDL and EPA as an atherogenic and anti-atherogenic factor is not fully understood; depending on its ligands, PPAR- γ may have protective or supportive influences on atherosclerosis [17].

In the present study, our aim was to investigate the expression level of PPAR- γ and CD36 in response to these two opposing factors (ox-LDL and EPA). Furthermore, PPAR- γ antagonist (T0070907) was employed to identify the role of PPAR- γ in expression of CD36 in macrophage stimulated by ox-LDL and EPA, and to understand whether other transcription factors apart from PPAR- γ play a role in expression of CD36 or not. T0070907 is a specific inhibitor with high selectivity for PPAR- γ that blocks its activity in both biochemical and cell-based assays. T0070907 covalently modifies one of the conserved residues, cys313, in PPAR- γ [18].

MATERIALS AND METHODS

Reagents. DMEM, fetal bovine serum, penicillin-streptomycin and serum-free medium (SFM) were

purchased from Invitrogen (Gibco, USA). T0070907, EPA and protease inhibitor cocktail were obtained from Sigma, St. Louis, USA, β -Actin, CD36 and PPAR- γ primers were purchased from QIAGEN, Germany). Primary antibody of CD36, PPAR- γ and β -actin and the secondary antibody were purchased from Abcam Inc, USA.

Cell culture. Raw 264.7, a murine macrophage cell line, was purchased from Genetic National Institute (Tehran, Iran). These cells were grown in DMEM culture medium supplemented with 10% heat-inactivated fetal bovine serum, 3 mM glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. All treatments were performed on SFM.

Preparation of oxidized low-density lipoprotein. LDL was isolated from human fresh plasma by sequential ultracentrifugation. Plasma was obtained from blood bank from normolipidemic blood donor volunteers. Each 100 ml plasma was supplemented with 1.6 ml EDTA 0.2 M, 2.8 ml NaCl 0.3 M, 10 mg chloramphenicol and protease inhibitor cocktail. Plasma density was then adjusted to 1.063 g/ml with potassium bromide (KBr). First, centrifugation was performed at 100,000 \times g at 4°C for 10 hours to separate very low density lipoprotein (VLDL), chylomicron and LDL from high density lipoprotein. The top orange layer consisted of LDL with creamy layer (VLDL and chylomicron) on top of it. LDL was separated from VLDL and chylomicron by centrifugation at 100,000 \times g for at 4°C 4 hours then the top orange layer was collected as LDL and dialyzed against PBS at 4°C for 24 hours. The concentration of LDL protein was determined by Bradford's assay [19].

Low-density lipoprotein oxidation. Purified LDL was oxidized by induction with Cu²⁺. For this reaction dialysis, a tube containing 10 mg/ml LDL protein was placed in PBS buffer containing 10 μ M Cu²⁺ at 37°C for 18 hours, then 200 μ M EDTA was used to stop oxidation and chelate the Cu²⁺ ion [19].

Analysis of low-density lipoprotein. Following isolation of LDL and modification with CuSO₄, electrophoretic migration in agarose gel was performed to assess the purity and extent of oxidation of LDL. The electrophoretic mobility was measured in agarose 1%, and then lipoprotein bands were detected by lipid-specific stain, Oil Red O [20].

Preparation of bovine serum albumin-conjugated eicosapentaenoic acid. EPA was dissolved in DMSO

and diluted in SFM containing 1% BSA to reach concentrations of 100 μ M and 200 μ M and then incubated at 37°C on rotary shaker for 2 hours.

MTT assay/cell viability: The cytotoxicity assay was used to measure cell viability and performed by reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, USA) with mitochondrial dehydrogenase of viable cells which produced a blue-colored formazan. The optical density at 540 nm was used to measure concentration of blue color. Murine macrophage cell lines were incubated with ox-LDL and EPA in 96-well plates. After 48 hours, the cells were incubated with 0.5 mg/ml MTT at 37°C for 4 hours, then dark blue formazan crystal was dissolved in 100 μ L DMSO at 37°C for 10 min. The optical density was measured at 540 nm with an ELISA reader [21].

Gene expression analysis: To measure gene expression, total mRNA was prepared from 4×10^5 cells in a 6-well plate using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Purity and integrity of RNA was qualified with spectrophotometry (260/280 nm) and agarose gel, respectively. cDNA was synthesized using Quanti tect reverse transcriptase kit (QIAGEN, Germany) according to the manufacturer's instructions. Total RNA (1 μ g) was used for cDNA synthesis. Quantitative real-time PCR was performed with Rotor-Gene real-time thermocycler and SYBR Green PCR Master Mix (QIAGEN, Germany). Primers of CD36, PPAR- γ and β -Actin as endogenous controls (housekeeping gene) were from (QIAGEN, Germany). The primer sequences are not available, but the manufacturer has verified their validity. To monitor the presence of non-specific products or primer dimer, melting curve was performed and run on 2% agarose gel. Relative quantitation of mRNA was estimated using comparative Ct (Δ Ct) method. The amplification efficiency of target gene and reference gene were calculated by LinReg PCR software and were approximately equal.

Western-blotting. Whole cell extraction was prepared after washing with cold PBS and then lysed with radio-immunoprecipitation assay buffer (150 mM sodium chloride, 1.0% NP-40 [nonyl phenoxypolyethoxyethanol] or Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 50 mM Tris, pH 8.0). Protein concentration of samples was determined by Bradford's assay. After separation on a 10% polyacrylamide gel (SDS-PAGE), proteins were transferred to the nitrocellulose membrane electrophoretically. Membrane was incubated with 3%

skim milk in TBS-T (Tris-buffered saline + 0.1% Tween 20) for 1 hour, followed by incubation with primary antibodies at 4°C overnight. The incubation time for secondary antibody (conjugated with horseradish peroxidase) was 1 hour at room temperature. After each step, membrane was washed 3 times with TBS-T for 5 min. The visualization of band was done by enhanced chemiluminescence (Perkin Elmer, Nederland). NIH Image J software was used to determine intensity of Western-blot bands [22].

Statistical analysis. Data are expressed as the mean \pm standard error. Differences among groups were analyzed by one-way analysis of variance (ANOVA) and followed by post hoc Tukey's test for sub-two group comparison. Statistical significance was considered as a *P* value < 0.05.

RESULTS

MTT Result. The MTT assay was used to determine toxic doses of EPA and ox-LDL. Figure 1 presents the results of the cytotoxicity effect of EPA and ox-LDL on Raw 264.7. The cells were treated with 10, 50, 100, 200 and 400 μ g protein/ml of ox-LDL. The two doses of ox-LDL (200 and 400 μ g protein/ml) were toxic for the cells (Fig. 2A). Therefore, 100 and 150 μ g protein/ml of ox-LDL were chosen for the following treatments. No toxic effect of EPA within the 50 to 500 μ M (50, 100, 200, 400 and 500 μ M) range was found on the macrophage cells (Fig. 2B). Murine macrophage cell line, Raw 264.7, was cultured in SFM overnight. The cells were treated with ox-LDL (100 and 150 μ g protein/ml) and EPA (100 and 200 μ M) for 24 and 48 hours in absence or presence of 2 μ M PPAR- γ inhibitor, T0070907.

Oxidized low-density lipoprotein stimulated expression of CD36. One of the main goals of this study was to investigate the signaling pathway that was involved in the expression of CD36. Therefore, the effect of ox-LDL on mRNA and protein expression of CD36 was first investigated. Our result showed that both mRNA and the protein level of CD36 were up-regulated by ox-LDL. CD36 mRNA was up-regulated by factors 11.8 ± 1.2 and 19.3 ± 1.6 in the sample group, which was treated by 100 and 150 μ g protein/ml of ox-LDL for 24 hours, respectively (Fig. 1A). Western-blotting experiment showed that ox-LDL induces 4.8 ± 0.9 and 9.49 ± 1.2 fold up-regulation of CD36 with 100 and 150 μ g protein/ml, respectively for 48 hours (Fig. 3A).

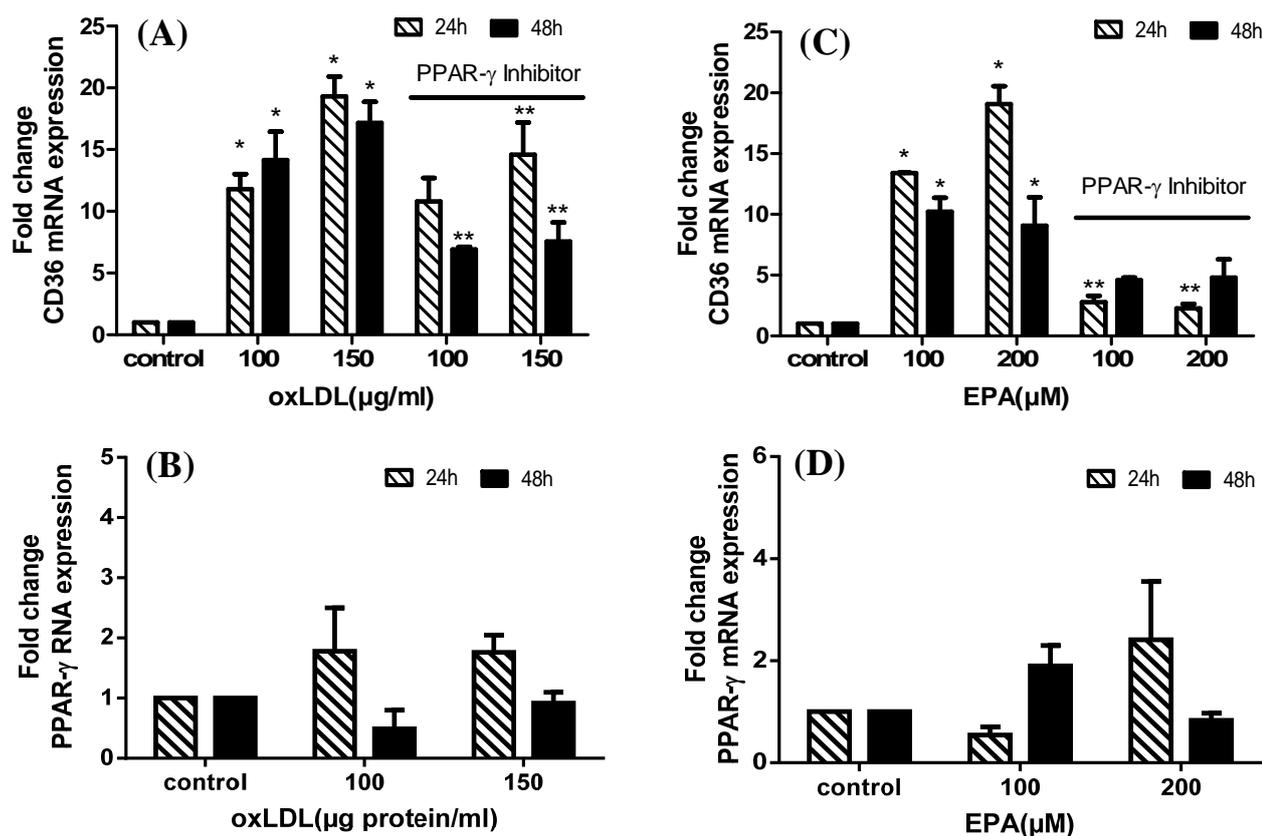


Fig.1. Effect of oxidized low-density lipoprotein (ox-LDL) and eicosapentaenoic acid (EPA) on CD36 and peroxisome proliferator-activated receptor gamma (PPAR- γ) mRNA in Raw264.7 cell line. Murine macrophage cell line (Raw264.7 cell line) was treated with 100 and 150 μ g protein/ml ox-LDL for 24 and 48 hours and separated group was pre-treated with 2 μ M T0070907 and then stimulated with ox-LDL. Also, Raw 264.7 cells were treated with 100 and 200 μ M EPA for 24 and 48 hours and separated group was pre-treated with 2 μ M T0070907. CD36 and PPAR- γ mRNA were analyzed with SYBR Green real-time PCR normalized against β -actin and expressed as relative to control. Data are presented as mean \pm S.E.M. * $P \leq 0.05$ relative to control. ** $P \leq 0.05$ relative to each corresponding ox-LDL. (A) Effect of ox-LDL on CD36 mRNA in absence or presence of inhibitor. (B) Effect of ox-LDL on PPAR- γ mRNA. (C) Effect of EPA on CD36 mRNA with and without inhibitor. (D) Effect of EPA on PPAR- γ mRNA with and without inhibitor.

Effect of oxidized low-density lipoprotein on expression of peroxisome proliferator-activated receptor gamma. The cells were harvested after 24 and 48 hours with 100 and 150 μ g protein/ml ox-LDL. Protein and mRNA levels of PPAR- γ slightly increased in comparison with control but did not change significantly (Fig. 1B and 3B). Considering the up-regulation of CD36 in the cells treated with ox-LDL, PPAR- γ expression was expected to be increased, but no significant increase was observed in the mRNA and protein level of PPAR- γ .

T0070907 (peroxisome proliferator-activated receptor gamma inhibitor) decreased CD36 expression. CD36 was stimulated by ox-LDL, and the expression level of PPAR- γ was not up-regulated significantly. In this step, an attempt was made to find out whether the increasing level of CD36 is dependent on up-regulation of PPAR- γ or its activity (by phosphorylation or by ligand); therefore, a specific

PPAR- γ inhibitor, T0070907, was used to explore the role of PPAR- γ in this pathway. Cells were pretreated with 2 μ M T0070907 for 2 hours. Ox-LDL at 100 and 150 μ g protein/ml concentrations for 24 and 48 hours effectively increased the mRNA and protein level of CD36. It is interesting to note that PPAR- γ inhibitor T0070907 decreased the protein and mRNA level of CD36 (Fig 1A). PPAR- γ inhibitor decreased CD36 protein expression from 9.49 to 2.25 folds in cells treated with 150 μ g protein/ml ox-LDL for 48 hours (Fig. 3A).

Eicosapentaenoic acid up-regulated expression of CD36. Murine macrophage cell line, Raw 264.7, was harvested after treatment with 100 μ M and 200 μ M EPA for 24 and 48 hours. Next, to further clarify the role of PPAR- γ , the PPAR- γ inhibitor was used and demonstrated that the PPAR- γ inhibitor T0070907 (2 μ M) decreased expression of protein and mRNA of CD36. Cells were pre-incubated with 2 μ M PPAR- γ

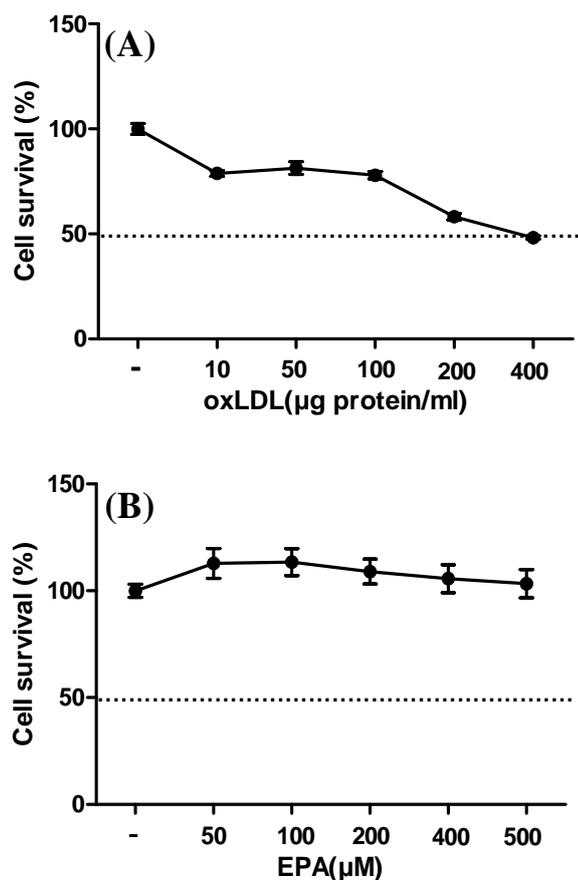


Fig. 2. MTT assay/cell viability in the presence of oxidized LDL (ox-LDL) and eicosapentaenoic acid (EPA). Raw 264.7 cells were incubated in serum-free medium containing EPA and ox-LDL in a 96-well plate to determine the toxic doses and after 48 hours cell viability was measured by MTT assay. The results are presented as percent of control response and mean \pm standard deviation. Graphs of (A) ox-LDL MTT and (B) EPA MTT.

inhibitor (T0070907) for 2 hours. Quantitative real-time PCR was used to determine CD36 mRNA levels in cells in absence or presence of 2 μ M T0070907 in response to EPA. In 24 hours, 100 μ M and 200 μ M EPA caused up-regulation of CD36 mRNA by a factor of 13.4 and 19, respectively (Fig. 1C). Also, the results of Western-blotting showed that CD36 protein was up-regulated by a factor of 7.97 ± 0.17 and 9.2 ± 0.4 in the cells that were treated with 100 μ M and 200 μ M EPA in 24 hours (Fig. 4A).

Eicosapentaenoic acid does not have a significant effect on expression of peroxisome proliferator-activated receptor gamma. No significant increase in PPAR- γ mRNA and protein levels was detected in the cells treated with EPA for 24 hours and 48 hours (Fig. 1D and 4B).

DISCUSSION

The main goal of the present study was to evaluate the effect of ox-LDL (atherogenic factor) and EPA (anti-atherogenic factor) on the expression of CD36 and PPAR- γ , and the role of PPAR- γ in expression of CD36 by using PPAR- γ inhibitor was investigated as well. Our data showed that CD36 expression was up-regulated by ox-LDL. While LDL down-regulated its receptor, ox-LDL up-regulated CD36 expression. It has been shown that ox-LDL enhanced promoter activity of the CD36 gene, which has PPAR- γ response element [15]. Our result is consistent with previous studies which revealed that CD36 is stimulated in macrophages by ox-LDL [23, 24].

Furthermore, we investigated the effect of EPA as an important member of the ω -3 fatty acid family on the expression of CD36. We demonstrated that EPA stimulated CD36 mRNA and protein, significantly. Although Pietsch *et al.* [25] reported that EPA down-regulated CD36 expression and mentioned that this is the protective role of ω -3 fatty acid in inhibition of atherosclerotic plaque formation, Wang *et al.* [26] reported that reduction of ratio of ω -6 PUFA (EPA plus DHA) led to reduced atherosclerotic lesion but had no effect on expression of CD36. Additionally, McLaren *et al.* [27] found that EPA and DHA produced a statistically significant increase in CD36 mRNA levels in human acute monocytic leukemia cell line (THP-1 macrophages). These two recent studies are in agreement with our study and confirm that anti-atherosclerotic effect of PUFA is not mediated through a decrease in CD36 gene expression.

It has been reported that PPAR- γ plays a critical role in regulation of CD36 and absence of PPAR- γ leads to very low expression of CD36 protein and mRNA [28]. Several studies showed that PPAR- γ has a role in expression of CD36 in the cells treated with ox-LDL [15, 23]. Since Ox-LDL and EPA caused up-regulation of CD36 in the present study, it was assumed that this was accomplished by increasing PPAR- γ gene expression. Nevertheless, no significant up-regulation of PPAR- γ mRNA and protein by ox-LDL and EPA was detected. Therefore, in the next step, we tried to find out how CD36 expression was increased without significant up-regulation of PPAR- γ . For this reason, PPAR- γ inhibitor was employed to clarify this transcription factor role in CD36 expression. PPAR- γ inhibitor was used and it was found that expression of CD36 was blocked in the presence of T0070907, which confirmed that PPAR- γ does have a role in signaling pathway of CD36 expression. Therefore, the results appear to indicate that activation of PPAR- γ (by ligand or phosphorylation) might have a greater responsibility in regulation of CD36 than in increasing expression of PPAR- γ .

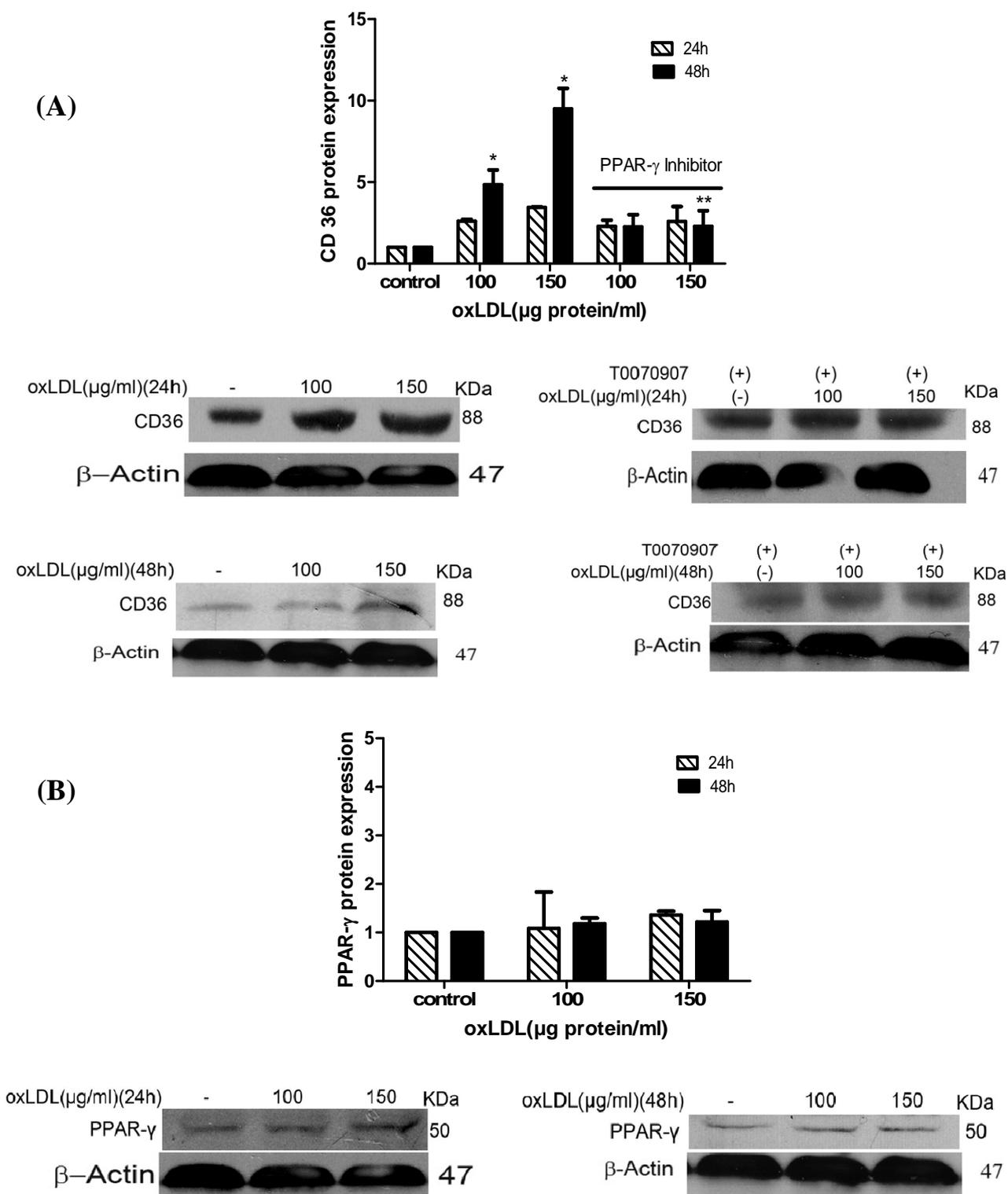


Fig. 3. Effect of ox-LDL on CD36 and peroxisome proliferator-activated receptor gamma (PPAR- γ) protein in Raw264.7 cell line. Murine macrophage cell (Raw 264.7 cell line) was treated with 100 and 150 μg protein/ml ox-LDL for 24 and 48 hours and separated group was pre-treated with 2 μM T0070907 and then stimulated with ox-LDL. Whole cell lysate was used for analysis with Western-blot to determine protein expression of CD36 and PPAR- γ . Specific CD36 and PPAR- γ antibodies were used. Semi-quantitative analysis was performed by using image J (NIH) for densitometry analysis and then normalized against β -actin as endogenous control and expressed as relative to control (DMSO). All results are presented as mean \pm S.E.M. * $P < 0.05$ relative to control. ** $P < 0.05$ relative to each corresponding ox-LDL. **(A)** Effect of ox-LDL on CD36 protein in absence or presence of 2 μM T0070907. **(B)** Effect of ox-LDL on PPAR- γ protein expression.

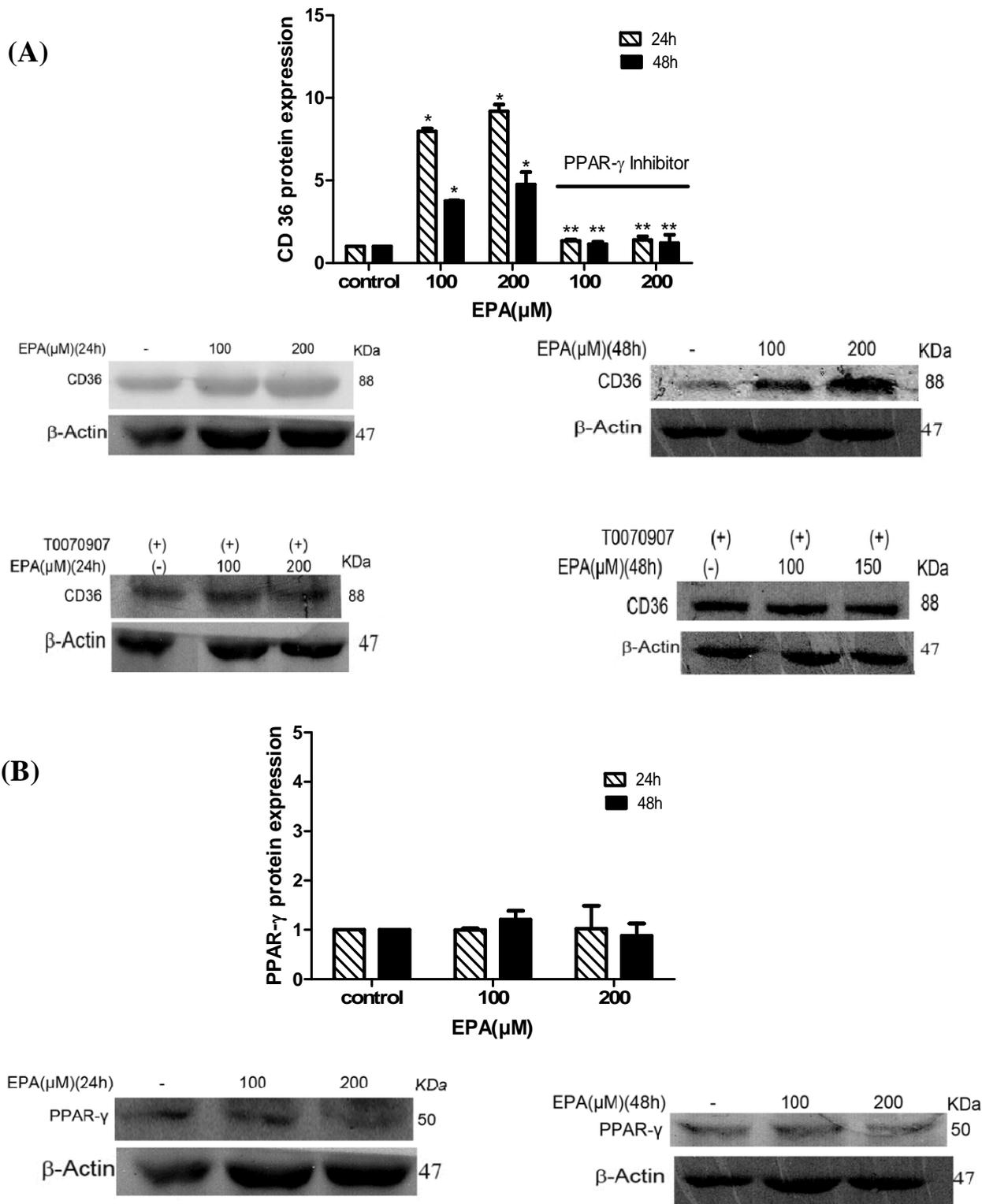


Fig. 4. Effect eicosapentaenoic acid (EPA) on CD36 and peroxisome proliferator-activated receptor gamma (PPAR- γ) protein in Raw264.7 cell line. Murine macrophage cell line (Raw 264.7 cell line) was treated with 100 and 200 μ M EPA for 24 and 48 hours and separated group was pre-treated with 2 μ M T0070907. Whole cell lysate was used for analysis with Western-blot to determine protein expression of CD36 and PPAR- γ . Specific CD36 and PPAR- γ antibodies were used. Semi-quantitative analysis was performed by using image J (NIH) for densitometry analysis and then normalized against β -actin as endogenous control and expressed as relative to control (DMSO). All results are presented as mean \pm S.E.M. * $P \leq 0.05$ relative to control. ** $P \leq 0.05$ relative to each corresponding EPA. **(A)** Effect of EPA on CD36 protein expression in absence or presence of 2 μ M T0070907. **(B)** Effect of EPA on PPAR- γ protein expression.

Rios *et al.* [29] reported that ox-LDL stimulated CD36, but in contrast to our result they indicated that this mechanism is not dependent on PPAR- γ . They discovered that GW9662 (another PPAR- γ inhibitor) could not inhibit expression of CD36 by ox-LDL. The important factor in this controversy might stem from different PPAR- γ inhibitors. Rios *et al.* [29] used GW9662 and demonstrated that in addition to PPAR- γ inhibition, it increases PPAR- α expression [30], which has been shown to be involved in stimulation of CD36 expression [31]. Consequently, activation of PPAR- α might have modulated CD36 expression. However, T0070907 is a PPAR- γ specific inhibitor and has no effect on expression of PPAR- α .

It has been shown that various PPAR- γ ligands can stimulate CD36 expression through activation of PPAR- γ [32, 33]. It has been also shown that activators of protein kinase C increased expression of CD36 via phosphorylation of PPAR- γ and also protein kinase C inhibitor decreased expression of CD36 [34].

It was concluded that as PPAR- γ inhibitor decreased expression of CD36, ox-LDL and EPA increased CD36 expression through increasing PPAR- γ activity not its expression.

Ishii *et al.* [35], for the first time, reported that in addition to PPAR- γ , another transcription factor called Nrf-2 plays a role in regulation of CD36 expression in murine macrophage. They showed that ox-LDL induced nuclear accumulation of Nrf-2 and resulted in stimulation of CD36 in macrophage.

This study does not exclude the possibility of some other contributing transcription factors such as Nrf-2 in regulation of CD36 expression, since our result showed that CD36 up-regulation did not return to the control level when treated with PPAR- γ inhibitor (T0070907).

The interesting point is that in the cells pretreated with PPAR- γ inhibitor, reduction of the EPA-induced increase of CD36 expression was more than the reduction of the ox-LDL-induced increase of CD36 expression. Also, This finding indicated that PPAR- γ has a more determinant role in expression of CD36 by EPA than ox-LDL. Therefore, PPAR- γ activation by EPA can stimulate both CD36 and ABC1 expression and might increase the efflux of cholesterol. Nevertheless, further studies are needed to evaluate this pathway.

We have shown that PPAR- γ plays a major role in CD36 regulation but it was found that induction of CD36 is not dependent on the increasing expression of PPAR- γ , and increasing PPAR- γ activity might play a role in stimulation of CD36 expression. It was also demonstrated that CD36 is not the target for ω -3 fatty acids (EPA) to exert their protective effect in formation of atherosclerotic plaque.

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