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Effects of palmitate and astaxanthin on cell viability and proinflammatory characteristics of mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) have broad immunomodulatory activities. These cells are a stable source of cytokine production such as interleukin-6 (IL6), monocyte chemoattractant protein-1 (MCP-1/CCL2) and vascular endothelial growth factor (VEGF). Fatty acid elevation in chronic metabolic diseases alters the microenvironment of MSCs and thereby, might affect their survival and cytokine production. In the present study, we investigated the effects of palmitate, the most abundant saturated free fatty acid (FFA) in plasma, and astaxanthin, a potent antioxidant, on cell viability and apoptosis in human bone marrow-driven mesenchymal stem cells. We also elucidated how palmitate and astaxanthin influence the inflammation in MSCs. Human mesenchymal stem cells were collected from an aspirate of the femurs and tibias marrow compartment. The effect of palmitate on cell viability, caspase activity and pro-inflammatory cytokines expression and secretion were evaluated. In addition, activation of the MAP kinases and NF-kB signaling pathways were investigated. The results showed that astaxanthin protected MSCs from palmitate-induced cell death. We found that palmitate significantly enhanced IL-6, VEGF and MCP-1 expression, and secretion in MSC cells. Increased cytokine expression was parallel to the enhanced phosphorylation of P38, ERK and IKKα-IKKβ. In addition, pretreatment with JNK, ERK, P38, and NF-kB inhibitors could correspondingly attenuate palmitate-induced expression of VEGF, IL-6, and MCP-1. Our results demonstrated that fatty acid exposure causes inflammatory responses in MSCs that can be alleviated favorably by astaxanthin treatment.

1. Introduction

The capability of mesenchymal stem cells (MSCs) to differentiate into multiple cell lineages could act as a reserve cell supply for tissue regeneration [1]. Additionally, these cells have been demonstrated to have immunoregulatory features. Recent studies have discovered that MSCs interfere with elements of the innate immune system to adopt anti-inflammatory or pro-inflammatory effects [2]. MSCs have also been shown to be a stable source of cytokines production, such as vascular endothelial growth factor (VEGF), interleukin-6, and monocyte chemoattractant protein-1 (MCP-1) [3]. VEGF is one of the main factors in the angiogenesis process, and recently, it has also been suggested that angiogenesis plays a crucial role in the pathophysiology of chronic inflammatory diseases [4]. MCP-1 as a known chemoattractant factor is involved in infiltration of inflammatory cells and performs multiple functions in low-grade inflammatory disease [5–9]. In this regard, MSCs are believed to release pro-inflammatory cytokines in response to inflammatory microenvironment changes [10]. Therefore, understanding the effect of different metabolic microenvironments on biological characteristics of stem cells could give rise to new possibilities for controlling the function of stem cells. Plasma free fatty acids (FFA) are elevated in most obese subjects. They are an important link between obesity, diabetes, and nonalcoholic fatty liver [11]. Although FFAs elevation cause detrimental metabolic effects and play an important role in the development of chronic inflammatory diseases, such as fatty liver and diabetes, less is known about their effects on the biological features of stem cells. The crucial character of stem cells is their ability to self-renew to produce more stem cells. Therefore, in the present study, we evaluated cell viability and caspase activation in MSCs exposed to palmitate. Furthermore, little is known about the effect of palmitate on MSCs cytokine secretion, thus, we investigated the effect of palmitate on pro-inflammatory cytokines secretion. Moreover, it is necessary to search for compounds that the can attenuate inflammatory phenotype in MSCs for promising stem cell therapy. Currently, natural compounds are receiving increasing attention in the development of drugs for the treatment of various diseases. Astaxanthin

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is a carotenoid pigment with antioxidant and anti-inflammatory properties. Antioxidant and anti-inflammatory properties of astaxanthin are unique based on its molecular structure that has specific hydroxyl and ketone moieties that explain its higher antioxidant activity compared to the other carotenoids [12,13]. Astaxanthin has also been shown to have various beneficial effects on cardiovascular diseases, insulin resistance, and excessive hepatic lipid accumulation and peroxidation [14–17]. Astaxanthin inhibits TNF-α, and IL-1β expression in lipopolysaccharide administrated mice. Astaxanthin suppresses NF-KB activity in RAW264.7 cells activated with lipopolysaccharide [18]. Therefore, in the present study, we also investigated the possible protective effect of astaxanthin on cell viability and inflammatory responses in palmitate-treated MSCs.

2. Materials and methods

2.1. Characterization and culture of MSCs

Human mesenchymal stem cells were kindly provided by the laboratory of Dr. Masoud Soleimani at the Department of Hematology, Tarbiat Modares University, Tehran, Iran. MSCs were cultured in DMEM medium supplemented with 10% FBS and 50 Unit/ml penicillin and 50 mg/ml streptomycin (Invitrogen, Carlsbad, USA). The plates were maintained at 37 °C in 5% CO2. The cells in third passage were used for all experiments. We confirmed that MSCs retain the expression of their characteristic cell surface markers, i.e., CD105, CD90, CD73, CD34, CD45, and CD19 following palmitate and astaxanthin treatments using flow cytometry (Table 1). The capacity of these cells to differentiate into adipocytes and osteoblasts were also examined as described in a previous publication [19].

2.2. Treatment of mesenchymal stem cells

Lipid-containing media was prepared as described by Chavez et al. [20]. Briefly, sodium palmitate (Sigma-Aldrich) was dissolved in 50% (v/v) ethanol. After filtration, the solutions were diluted with pre-warmed DMEM containing 1% (w/v) fatty acid- free BSA to the final concentration and incubated in 37 °C for 2 h, along with shaking. Palmitate treatments were performed with the concentration of 0.5 mM for 24 h [21,22]. Two hours before the experiments, the media were replaced with serum-free-DMEM containing 1% BSA and then the cells were incubated in the presence or absence of 0.5 mM lipid-containing media for 24 h. For astaxanthin pretreatment (10 μM), it was added 30 min before the incubation with lipid-containing media. In order to elucidate the signaling mediators of palmitate effects, specific inhibitors of JNK (SP600125, 20 μM), ERK (PD98059, 20 μM), p38 MAPK (SB2021190, 10 μM), and NF-κB (parthenolide, 20 μM) were added to cell culture media 2 h before incubation with palmitate.

2.3. Cell viability assay

CellTiter - Blue Cell Viability Assay (Promega, Madison, WI, USA) was used to measure cell viability, according to the manufacturer's instructions. Briefly, this assay was based on the conversion of resazurin to the fluorescent molecule, resorufin. The fluorescent molecule was detected at the excitation wavelength of 544 nm and an emission wavelength of 590 nm that was proportional to the number of viable cells.

Table 1
Surface markers of human mesenchymal stem cell following 24 h palmitate exposure.

Cell surface marker	Control MSCs*	Palmitate treated MSCs*
CD73	98.61 ± 0.71	97.1 ± 0.41
CD90	99.21 ± 0.34	98.9 ± 0.35
CD105	99.1 ± 0.27	98.8 ± 0.25
CD19	0.05 ± 0.05	0.07 ± 0.07
CD34	0.06 ± 0.04	0.08 ± 0.02
CD45	0.28 ± 0.21	0.19 ± 0.3

* Percentage of the cells positive for a specific cell surface marker (n = 3). There was no significant difference between control and palmitate treated cells.

2.4. Caspase activity assay

After the treatments, MSCs were subjected to Caspase-3/7 activity assay using the Caspase-Glo® 3/7 assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Caspase-Glo® 3/7 assay Kit is a luminescent assay that measures Caspase-3/7 activities. The sample luminescence was measured using a plate reader luminometer (Thermo Labsystems) at the excitation wavelength of 490 nm and an emission wavelength of 520 nm.

2.5. Determination of pro-inflammatory cytokines concentration in cell culture supernatants

Following treatments, the cell culture supernatants were collected and stored at −80 °C for IL6, VEGF, and MCP-1 determination. The concentrations were determined using respective ELISA kits (R&D Systems Inc., MN, USA), following the manufacturer's instructions.

2.6. Gene expression analysis

Total RNA was extracted from the treated MSCs using the RNeasy mini kit (Qiagen, MD, USA). Reverse transcription was performed with 1 μg RNA using the Qiagen reverse transcriptase and random hexamer primers. IL-6, VEGF, and MCP-1 expression levels were measured by the Applied Biosystem 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the company's proprietary master mix and primer sets (Applied Biosystems, Foster City, CA, USA). The expression of VEGF, IL-6, and MCP-1 mRNA were normalized to the beta-actin expression. The mean value of triplicate measurements was used for the comparison of the mRNA levels.

2.7. Western blot analysis

Western blotting was applied to evaluate the effect of palmitate in the presence and absence of astaxanthin on inflammatory signaling pathways. RIPA buffer supplemented with protease inhibitor (Roche, Mississauga, ON, Canada) was used to prepare cell lysate. Western blotting was conducted as previously described [23]. The applied antibodies included anti-JNK, anti- p-JNK, anti-P38, and IKKα-IKKβ (all from Cell Signaling Technology, Beverly, MA, USA) and anti-b-actin (Abcam, Cambridge, MA, USA). A horseradish peroxidase-conjugated antibody was used as the secondary antibody and protein bands visualized using an enhanced chemiluminescence substrate (ECL) according to manufacturer's protocol.

2.8. Statistical analysis

Data from each group are presented as the mean ± SEM of at least three separate measurements. The data were analyzed using the SPSS software (SPSS 22, Chicago, IL, USA). Statistical comparisons between the groups were performed using one-way or two-way analysis of variance (ANOVA) followed by Tukey post hoc tests. Differences with $P < 0.05$ considered statistically significant.

3. Results

Astaxanthin protects mesenchymal stem cells from palmitate-induced cell death.

Palmitate treatment as indicated in Fig. 1A resulted in a significant decrease in the number of viable cells. Treatment with 0.5 mM

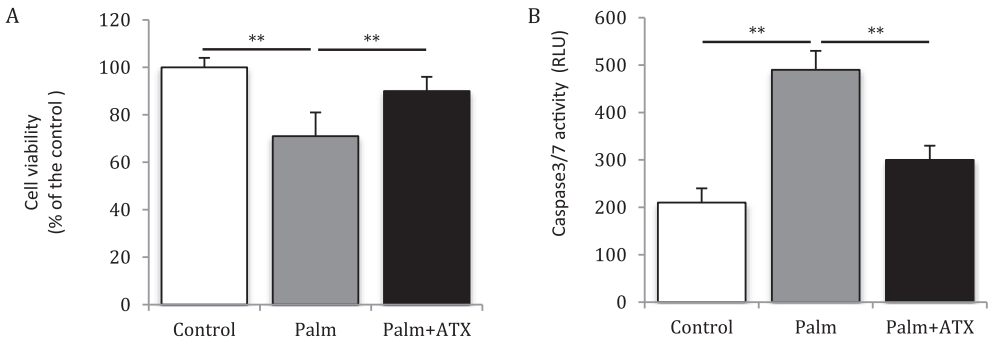

Fig. 1. Astaxanthin protects mesenchymal stem cells from palmitate-induced cell death. The control group was cultured in fatty acid-free medium. The palmitate group was treated with 0.5 mM palmitate complexed with BSA (Palm) and the Palm + ATX group received 10 μM astaxanthin prior to palmitate ($n = 3$). (A) Cell viability was determined with CellTiter-Blue reagent; % viability was expressed as the percentage of viable cell in the treated groups to the control group. (B) Combined caspase 3/7 assays were performed using the Caspase 3/7-Glo kit after 24 h. ** $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

palmitate reduced cell viability, whereas, astaxanthin significantly prevented the harmful effects of palmitate.

To evaluate apoptosis, caspases-3/7 activity was measured. Palmitate increased caspases-3/7activity in MSCs. In contrast, palmitate-induced apoptosis was significantly suppressed in the presence of astaxanthin (Fig. 1B).

3.1. Palmitate-induced pro-inflammatory cytokines production was inhibited by astaxanthin

In the present study, we evaluated the secretion and expression of three MSCs derived cytokines, including VEGF, IL-6, and MCP-1 after treatment with 0.5 mM palmitate. The secretion and expression of VEGF, IL-6, and MCP-1 were increased significantly. But, pretreatment of MSCs with 10 μM astaxanthin significantly attenuated the palmitate effects on VEGF, IL-6, and MCP-1 secretion and expression (Fig. 2.)

3.2. MAPK pathways are involved in palmitate induced-cytokine secretion

The MAP kinases are among the most important signaling pathways involved in modulating cytokine production. The MAPKs include three main families, ERKs (extracellular-signal-regulated kinases), JNKs (c-Jun N-terminal kinases), and p38/SAPKs (stress-activated protein kinases). To investigate the cellular mechanisms underlying palmitate-induced cytokine secretion, we evaluated the possible role of MAPK signaling pathways. At first, phosphorylation of JNK, ERK, and p38 were assessed in the presence of 0.5 mM palmitate for 24 h. Our results indicated that palmitate treatment led to the activation of the MAPKs as demonstrated by increased phosphorylation of JNK, ERK, and p38 (Fig. 3.). Then, we confirmed the role of MAPKs signaling in palmitate-induced cytokine secretion by incubating MSCs with specific inhibitors of JNK (SP600125, 20 μM), ERK (PD98059, 20 μM), and p38 MAPK (SB2021190, 10 μM) for two hours prior to the palmitate treatment. As indicated in Fig. 3, we found that expression of VEGF, IL-6, and MCP-1 were attenuated in the presence of JNK, ERK, and P38 inhibitors.

3.3. NF-kB pathway is involved in palmitate induced-cytokine secretion

The transcription factor NF-kB pathway has been considered a major signaling pathway in induction of pro-inflammatory genes. To investigate NF-kB involvement in the palmitate-induced pro-inflammatory cytokine expression, we evaluated the NF-kB activation by detection of phosphorylation of NF-kB inhibitor IKKα-IKKβ. We found palmitate-induced phosphorylation of IKKα-IKKβ as shown in Fig. 4. In addition, NF-kB involvement in the cytokines release was further confirmed by parthenolide (20 μM) pretreatment, which significantly abolished palmitate-induced cytokine expression.

4. Discussion

Previous studies have clearly indicated that people with obesity, type 2 diabetes, and obesity-associated type 2 diabetes have higher plasma FFA levels [24]. Chronic exposure to saturated FFA leads to various metabolic alterations in the microenvironment that may affect MSCs properties and functions [18,25]. A defining characteristic of all stem cells is the ability to self-renew by producing more stem cells [26]. In this regard, we evaluated the effect of palmitate on viability of human MSCs. In line with previous studies in different cell types including MSCs [21,27], our results indicated that the chronic exposure of MSCs to the saturated FFA palmitate resulted in lipotoxicity, further leading to cell death. This finding suggests that palmitate as the most abundant FFA in plasma, might affect MSCs population that play a crucial role in maintaining tissue homeostasis. In contrast, we showed that astaxanthin protected MSCs against palmitate-induced apoptosis. The protective effects of astaxanthin have been reported in several other cell types [28]. However, to our knowledge, this is the first study showing a protective effect of astaxanthin on palmitate-induced cell death in human MSCs.

Additionally, it has been demonstrated that MSCs have broad immunomodulatory activities and are capable of influencing immune responses. Recent studies have shown that MSCs have the ability to adopt inflammatory or anti-inflammatory phenotypes in response to their microenvironments that is important for understanding their therapeutic potential in immune-mediated disorders [2]. It has been shown that MSCs constitutively secrete several mediators to the culture medium [2]. On the other hand, it has also been reported that VEGF, IL6, and MCP-1 expression are increased in chronic inflammatory diseases such as obesity, diabetes, and fatty liver and there is a link between disease progression, inflammation, and angiogenesis [29–32]. In accordance to these findings, results of the present study showed that palmitate upregulated the expression and release of MSCs derived inflammatory mediators. VEGF, IL-6 and MCP-1 are known as the main contributors to chronic inflammation via enhancing recruitment and migration of immune cells into the tissues [33]. Thus, MSCs may play a role in augmenting inflammation in high palmitate conditions. In addition, we found that astaxanthin inhibits the effects of palmitate on VEGF, IL-6, and MCP-1 expression. Elevation of these cytokines can trigger inflammation, while astaxanthin may protect the tissue from inflammation via inhibition of palmitate-induced VEGF, IL-6, and MCP-1 expression.

To investigate the mechanism underlying palmitate-induced inflammation in MSCs, we evaluated the NF-κB and mitogen-activated protein kinases (MAPKs) pathways, which have been suggested as the key modulators of pro-inflammatory mediators production [34,35]. MAPK family consists of at least three classes in mammals' cells, the extracellular signal-regulated kinases (ERKs) and the two stress-

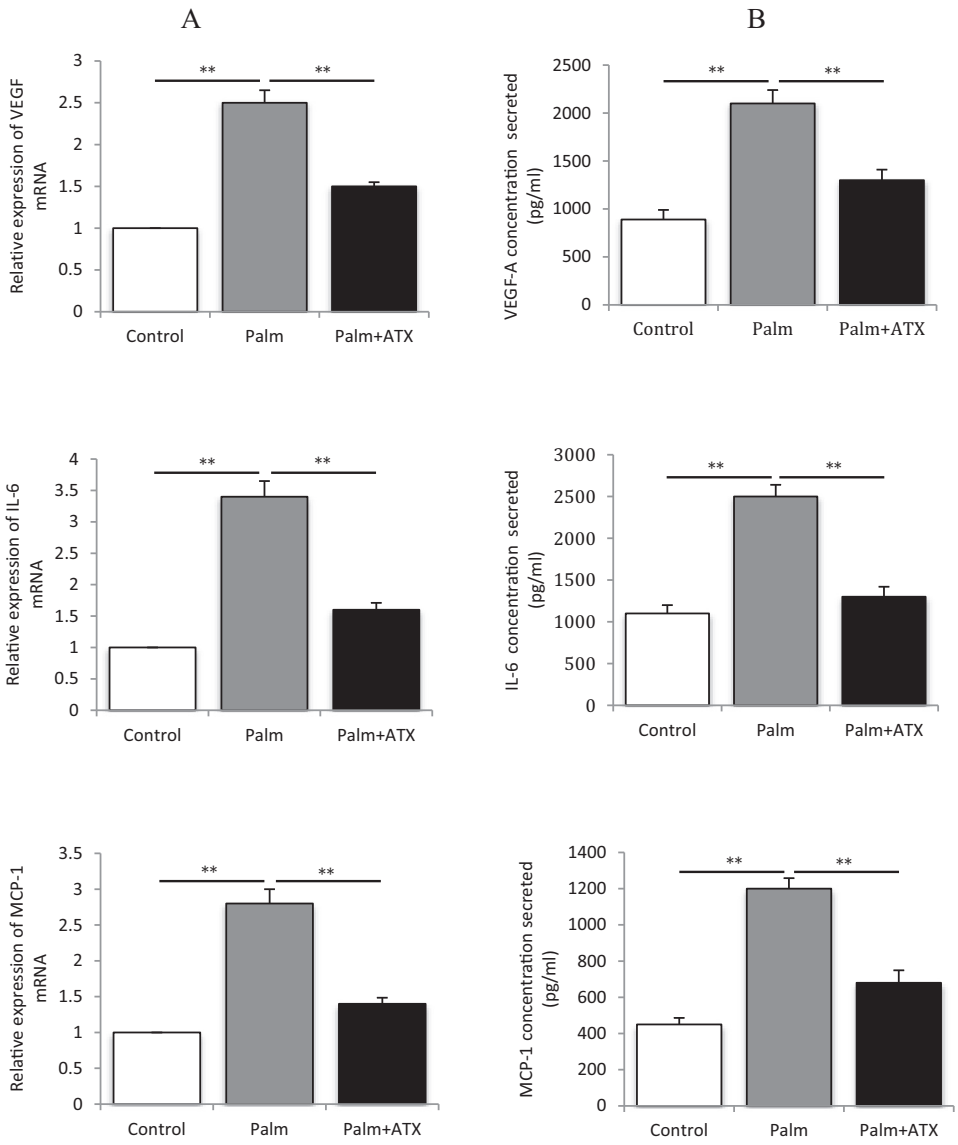

Fig. 2. Palmitate-induced pro-inflammatory cytokines production was inhibited by astaxanthin. The control group was cultured in fatty acid-free medium. The palmitate group was treated with 0.5 mM palmitate complexed with BSA (Palm) and the Palm + ATX group received 10 μM astaxanthin prior to palmitate ($n = 3$). (A) The gene expression of VEGF, IL-6, and MCP-1 were measured using real-time PCR and normalized to the beta-actin. (B) Cell culture supernatants were collected for assessment of cytokines secretion by ELISA. ** $p < 0.01$.

activated protein kinase (SAPKs) families, c-jun N-terminal kinase (JNK), and p38 [36]. We showed that palmitate led to the activation of MAPK pathways in MSCs, as demonstrated by increasing phosphorylation of JNK, P38, and ERK. These results are consistent with earlier studies that showed palmitate activate MAPK signaling in MSCs [22]. We confirmed the role of these pathways by pre-incubating MSCs with specific inhibitors of JNK, ERK, and p38 MAPK before palmitate stimulation. We found that palmitate induction of VEGF, IL-6, and MCP-1 was dependent on JNK, ERK and P38 MAPK pathways. In comparison, P38 and ERK were more noticeably induced in response to the palmitate treatment and their pharmacologic inhibition showed more potentials in suppressing cytokines expression, compare to the JNK. Moreover, our results showed that palmitate activates NF-κB transcription factor via increasing phosphorylation of IKKα-IKKβ. Taken together, these data suggest that palmitate induces pro-inflammatory mediators via different signaling pathways, i.e., MAPK, and NF-κB pathways, in MSCs.

Growing bodies of evidence have shown that astaxanthin is a potent anti-inflammatory and anti-apoptotic agent. AXT has been reported to exert its anti-inflammatory effect via inhibition of NF-κB-dependent signaling and gene expression of downstream inflammatory mediators [37]. In agreement with these studies, we found that pretreatment with ATX reduces palmitate-induced inflammation in MSCs. This is the first study that showed AXT inhibited NF-κB by decreasing IKKα-IKKβ phosphorylation and down-regulating transcription and secretion of VEGF, IL-6, and MCP-1 inflammatory mediators in human MSCs.

Our results indicated that chronic fatty acid exposure give rise to the inflammatory phenotype in MSCs whereas; AXT pretreatment could additionally attenuate palmitate-induced P38, ERK and NF-κB activation to alleviate inflammation. These findings suggest that a cautious approach is needed in stem cell therapy, given the potential of MSCs to enhance pro-inflammatory responses in a pathological state, and a possible outcome of applying MSCs may be increased disease progression [12,38]. Therefore, it is reasonable to apply anti-inflammatory compounds such as astaxanthin to relieve MSCs-derived inflammation via down-regulation inflammatory signaling pathways and cytokines expression.

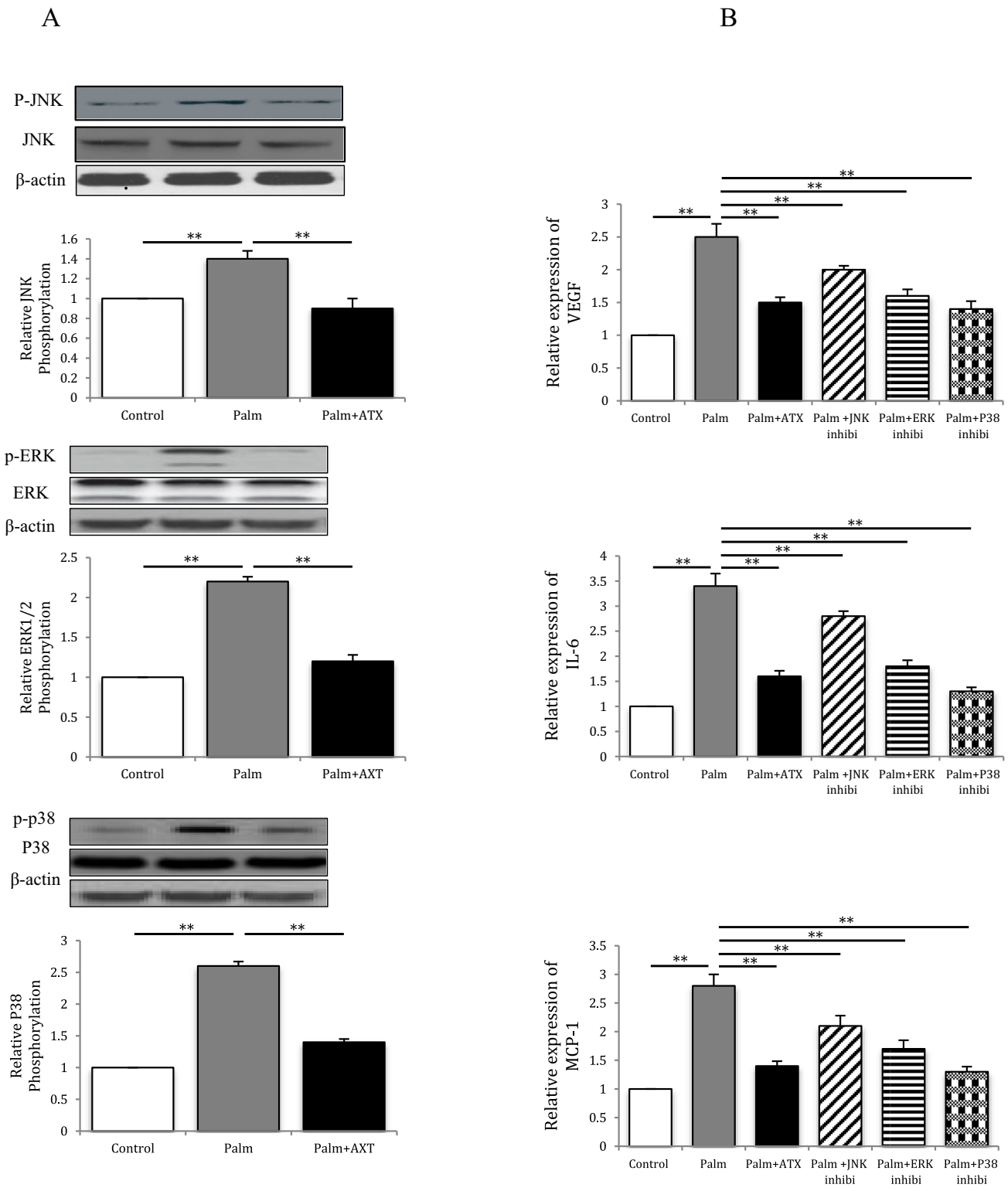

Fig. 3. MAPK pathways are involved in palmitate induced-cytokine secretion The control group was cultured in fatty acid-free medium. The palmitate group was treated with 0.5 mM palmitate complexed with BSA (Palm) and the Palm + ATX group received 10 μM astaxanthin prior to palmitate (n = 3). (A) Representative blots indicate the influence of palmitate on phosphorylation of JNK, ERK, and p38. (B) MSCs were also incubated with palmitate in the presence of JNK inhibitor (SP600125) (20 μM), P38 inhibitor (SB202190) (10 μM), and ERK inhibitor (PD98059) (20 μM) and IL-6, VEGF and MCP-1 expressions were determined by real time PCR. ** $p < 0.01$.

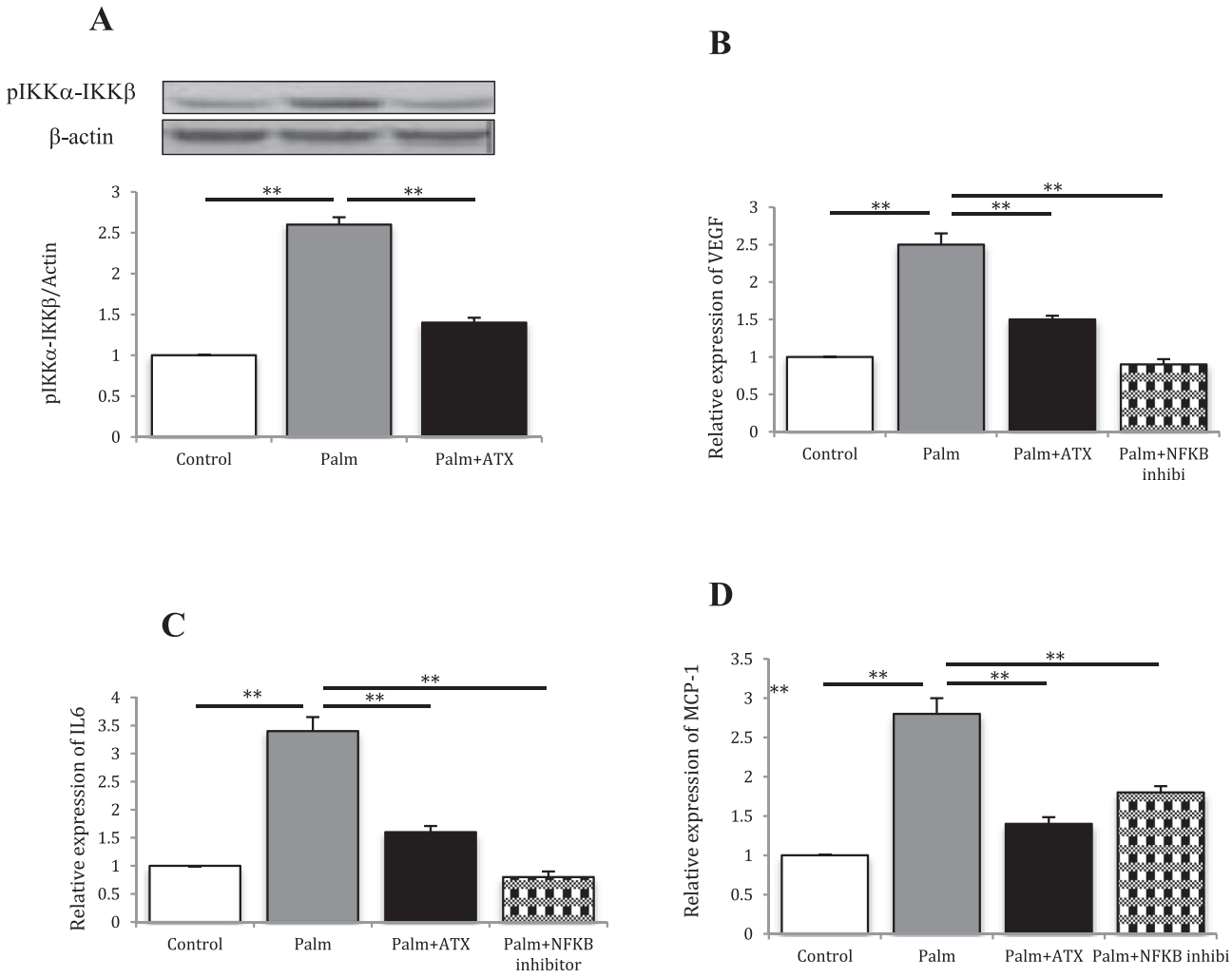

Fig. 4. NF-kB pathway is involved in palmitate induced-cytokine secretion. The control group was cultured in fatty acid-free medium. The palmitate group was treated with 0.5 mM palmitate complexed with BSA (Palm) and the Palm + ATX group received 10 μM astaxanthin prior to palmitate (n = 3). (A) Representative blots indicate the effect of palmitate on phosphorylation of IKKb/IKKa. MSCs were also incubated with palmitate in presence of NF-KB inhibitor and VEGF (B) IL-6 (C), and MCP-1(D) expressions were determined by real time PCR. ** $p < 0.01$.

Conflict of interest

The authors have declared no conflict of interest.

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