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Apolipoprotein A-I expression suppresses COX-2 expression by reducing reactive oxygen species in hepatocytes

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ABSTRACT

Abnormal lipid metabolism may contribute to the increase of reactive oxygen species (ROS) and inflammation in the pathogenesis of non-alcoholic steatohepatitis (NASH). Apolipoprotein A-I (apoA-I) accepts cellular cholesterol and phospholipids transported by ATP-binding cassette transporter A1 to generate nascent high density lipoprotein particles. Previous studies revealed that the overexpression of ABCA1 or apoA-I alleviated hepatic lipid levels by modifying lipid transport. Here, we examined the effect of apoA-I overexpression on ROS and genes involved in inflammation in both BEL-7402 hepatocytes and mice. Human apoA-I was overexpressed by transfection in BEL-7402 hepatocytes and by an adenoviral vector in C57BL/6J mice fed a methionine choline-deficient diet. The overexpression of apoA-I in both models resulted in decreased ROS and lipid peroxidation levels, as well as a reduced MAPK phosphorylation and decreased expression levels of c-Fos and COX-2. These results suggest that apoA-I overexpression can reduce steatosis by decreasing ROS levels and suppressing COX-2-induced inflammation in hepatocytes. MAPK and c-Fos are involved in this regulatory process.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an emerging metabolic disorder characterised by fatty infiltration of the liver in the absence of chronic alcohol consumption and macrovesicular steatosis. NAFLD is considered to be a hepatic manifestation of insulin resistance and metabolic syndrome [1–3]. Some patients may develop non-alcoholic steatohepatitis (NASH), which is characterised by superimposed ballooned hepatocytes, Mallory bodies, and lobular inflammatory cell infiltration. The pathogenesis of NASH is explained by the "two-hit" hypothesis [4], in which the first "hit" is steatosis and the second "hit" is the presence of oxidative stress and inflammation [5].

Apolipoprotein A-I (apoA-I), the primary protein component of HDL, functions by accepting cellular cholesterol and phospholipids transported by ATP-binding cassette transporter A1 (ABCA1) as the initial step of reverse cholesterol transport [6]. The atheroprotective effects of HDL, apoA-I, and apoA-I mimics have been demonstrated to extend beyond their role in reverse cholesterol transport to also include their anti-inflammatory and antioxidant activities [7]. Incubation of mesenchymal stem cells with HDL resulted in decreased reactive oxygen species (ROS) [8]. Endogenous apoA-I suppressed ovalbumin-induced neutrophilic airway inflammation [9]. An apoA-I mimetic peptide displayed anti-inflammatory and antioxidant properties both *in vivo* and *in vitro* [7]. Infusion of reconstituted HDL or apoA-I attenuated the pro-oxidant and proinflammatory changes induced by a periarterial collar in normocholesterolemic rabbits [10]. Currently, it remains unclear whether some of these effects of HDL and apoA-I are strictly due to functions of reverse cholesterol transport or whether these functions involve interactions with circulating proteins.

Most cells in the body produce prostaglandins as paracrine and autocrine mediators [11]. Cyclooxygenase-2 (COX-2) is an important enzyme in the pathway that converts arachidonic acid to prostaglandins. COX-2 can be induced by cytokines and growth factors, particularly at sites of inflammation and neoplasia [12]. Therefore, COX-2 plays a key role in inflammation and cancer [13]. It was reported that the induction of COX-2 during pancreatic cancer cell invasion was dependent on an extracellular

Abbreviations: ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; BSA, bovine serum albumin; ER, endoplasmic reticulum; DMEM, Dulbecco's minimum essential medium; HDL, high density lipoprotein; NASH, non-alcoholic steatohepatitis; COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase.

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signal-regulated kinase (ERK)/Ets-1-dependent mechanism [14]. COX-2 expression has also been linked to ROS; LPS-induced ROS was shown to be connected to AP1 activation and COX-2 induction [15].

Previous studies suggested that apoA-I expression promotes the clearance of reverse cholesterol transport from hepatocytes, reduces hepatic lipid accumulation, prevents ER stress, and suppress fatty acid synthesis [16–18]. In the current study, we examined the effects of apoA-I on fatty acid synthesis in the human hepatocyte cell line BEL-7402 and in C57BL/6J mice fed a methionine choline-deficient (MCD) diet. We showed that overexpression of apoA-I significantly reduced cellular ROS levels and suppressed the expression of pro-inflammatory gene COX-2 both *in vitro* and *in vivo*. These results suggest that an increased supply of apoA-I may be beneficial for the prevention and treatment of NASH.

2. Materials and methods

2.1. Materials

BEL-7402 cells were obtained from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA, USA). Fatty acid-free bovine serum albumin, protease inhibitor cocktail, linoleic acid (LA), H_2O_2, and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, USA). Triton X-100 was purchased from Beijing Solarbio (Beijing, China) and FuGene HD was obtained from Roche (Basel, Switzerland). Antibodies against ERK and COX-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against c-Fos was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Shanghai Kang Chen Biotech (Shanghai, China).

2.2. Cell culture

BEL-7402 cells were maintained in DMEM containing 10% foetal bovine serum or were incubated in serum-free DMEM with 1 mg/ml fatty acid-free bovine serum albumin (DMEM/BSA). Washed cells were incubated for 16 h in medium containing 5 mg/ml BSA in the presence or absence of 250 μM LA. LA bound to BSA at a 3.5 molar ratio was added from a stock solution [19].

2.3. Western blotting

Cells were washed and dislodged from the dish at 0 °C in a buffer containing protease inhibitors. Cellular proteins were solubilised in phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors and were then resolved by SDS–PAGE. The expression levels of ERK, c-Fos, and COX-2 were determined by Western blot analysis using the appropriate antibodies [20].

2.4. Measurement of intracellular ROS

BEL-7402 cells were cultured in 96-well plates and treated with or without 125 μM LA for 16 h. The cells were washed with PBS (pH 7.4) twice and then incubated with 100 μM DCF-DA for 30 min at 37 °C. The cells were then washed with PBS and the fluorescence intensity of intracellular 2',7'-dichlorodihydrofluorescein (DCF) was measured using Thermo Fluoroskan Ascent FL (Waltham, MA, USA). The excitation and emission wavelengths were 485 nm and 530 nm, respectively.

2.5. Assessment of lipid peroxidation

Malondialdehyde (MDA), one of the major secondary products of lipid peroxidation, was used to assess the levels of lipid peroxidation. The levels of MDA in BEL-7402 cells and mouse livers were assayed using the OxiSelect TBARS assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. A PerkinElmer Lambda 35 spectrophotometer (Waltham, MA, USA) was used for measurement.

2.6. Animal studies

C57BL/6J mice were purchased from the Academy of Military Medical Sciences (Beijing, China). Male mice (4–6 weeks old) were used in these studies. Before being fed an MCD diet, the mice were injected with 1×108 pfu/ml of an adenoviral vector containing human apoA-I (apoA-I) or an empty vector (Null) into the femoral vein. The mice were divided into four groups and were fed chow or the MCD diet for 1 week with or without the injection of the adenoviral vector. Experimental research on mice has been approved by the ethics committee in Capital Medical University. Animal studies also conform to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

2.7. Statistical analyses

The results of multiple observations are presented as the mean ± SEM. The data were analysed with the SPSS 11.5 statistics software (SPSS Inc., Chicago, IL, USA) using a nonparametric analysis of variance test. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Alteration of cellular ROS levels by apoA-I overexpression in BEL-7402 cells

Peroxidation of polyunsaturated fatty acids can result in increased cellular ROS levels [21]. Alterations to apoA-I expression levels may have a significant impact on cellular lipid levels. We increased apoA-I and ABCA1 expression levels in cholesterol- and oleic acid-treated BEL-7402 cells by transfecting the cells with plasmids expressing apoA-I. The overexpression of apoA-I resulted in a significant decrease in linoleic acid (LA)- or H_2O_2-induced ROS levels, as measured by the fluorescence intensity of DCF (Fig. 1A). We also measured the levels of MDA, an important marker indicating the level of lipid peroxidation (Fig. 1B). Consistent with the role of apoA-I as a lipid acceptor, apoA-I overexpression caused a significant decrease in cellular MDA levels compared with cells transfected with the vector alone. These data indicate that alterations of apoA-I expression levels lead to a change in cellular ROS levels.

3.2. Alteration of COX-2 expression by apoA-I overexpression in BEL-7402 cells

Because ROS can activate inflammation through different signalling pathways, we examined the possibility that ERK plays a role in ROS-induced inflammation in hepatocytes. BEL-7402 cells were transfected with an apoA-I expressing plasmid vector in the presence or absence of linoleate for 16 h, and the levels of phosphorylated and total ERK were measured by Western blot (Fig. 2A). While the levels of total ERK remained similar, the levels of phosphorylated ERK were significantly increased by LA; this increase was reduced by the overexpression of apoA-I. A similar expression pattern was also observed for the levels of c-Fos, a

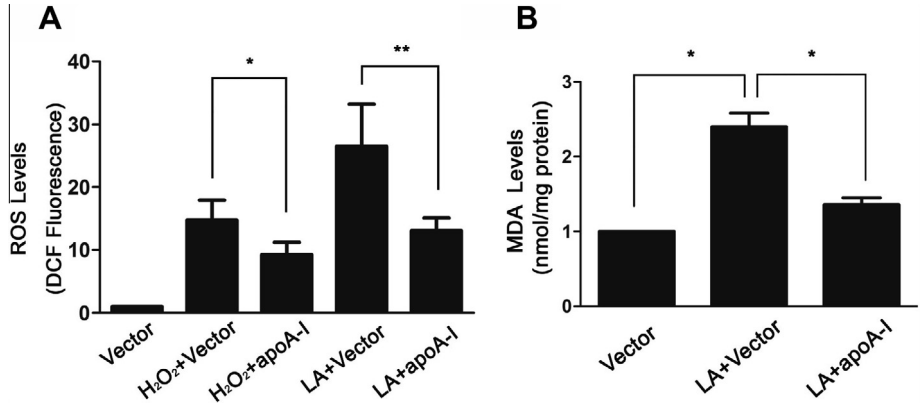

Fig. 1. ApoA-I overexpression alters cellular ROS levels in BEL-7402 cells. BEL-7402 cells were incubated for 24 h in DMEM containing 5 mg/ml BSA in the presence or absence of 250 μM LA or 0.6 mM H_2O_2. Vectors containing CMV alone or plasmids with the CMV-apoA-I constructs were then transfected using the FuGene HD transfection reagent. Forty-eight hours after transfection, (A) the cells were incubated with 100 μM DCF-DA for 30 min and the fluorescence intensity of intracellular DCF was then measured, and (B) MDA levels were assayed using the OxiSelect TBARS assay kit. The results are representative of three independent experiments and are presented as the mean ± SEM. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

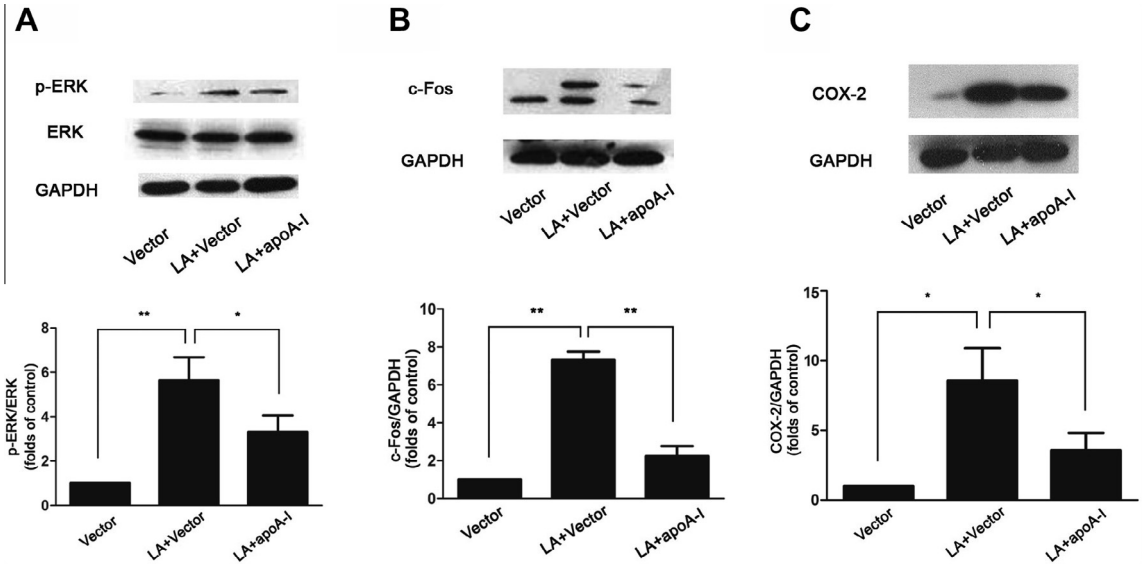

Fig. 2. ApoA-I overexpression decreases MAPK phosphorylation and c-Fos and COX-2 expression levels in BEL-7402 cells. BEL-7402 cells were incubated for 24 h in DMEM containing 5 mg/ml BSA in the presence or absence of 250 μM LA. Vectors containing CMV alone or plasmids with the CMV-apoA-I constructs were then transfected using the FuGene HD transfection reagent. Forty-eight hours after transfection, total cellular proteins were isolated and analysed for p-ERK, ERK (A), c-Fos (B), and COX-2 (C) by Western blotting. The results are representative of three independent experiments and are presented as the mean ± SEM. $^{*}P < 0.05$ versus control.

transcriptional factor regulated by ERK (Fig. 2B). Because c-Fos can activate COX-2 expression, we then measured the cellular levels of COX-2 under the same conditions described above (Fig. 2C). As predicted, the overexpression of apoA-I significantly reduced LA-induced COX-2 expression. These results further support the hypothesis that apoA-I overexpression reduces ROS levels and decreases COX-2 expression via the ERK and c-Fos pathway.

3.3. Alteration of ROS and COX-2 expression levels by apoA-I overexpression in vivo

To test the effects of apoA-I overexpression on ROS levels *in vivo*, we injected mice with either a control or apoA-I-expressing vector and exposed the mice to an MCD diet for 1 week. The MCD diet caused an increase in hepatic lipid [17] and MDA levels (Fig. 3); overexpression of apoA-I at least partially reversed this change. Consistent with the results from cultured cells, the expression of apoA-I *in vivo* significantly reduced the levels of phosphorylated ERK, as well as the expression levels of c-Fos and COX-2 that were induced by the MCD diet (Fig. 4A–C). These data further support the hypothesis that apoA-I overexpression can reduce ROS levels and suppress COX-2 expression through the ERK and c-Fos pathway.

4. Discussion

Modulation of apoA-I activity may have a beneficial effect on NASH. Our previous studies revealed that increased expression of apoA-I effectively regulated hepatic fatty acid and triglyceride contents and alleviated ER stress [17]. Here, we provide evidence that apoA-I overexpression may reduce inflammation by reducing ROS levels and suppressing COX-2 expression. In BEL-7402 cells, ROS and lipid peroxidation levels were significantly decreased upon apoA-I overexpression. ApoA-I overexpression also resulted in decreased ERK phosphorylation and c-Fos and COX-2 expression levels; a similar effect was observed when apoA-I was over-expressed in mice fed an MCD diet. These observations reveal an

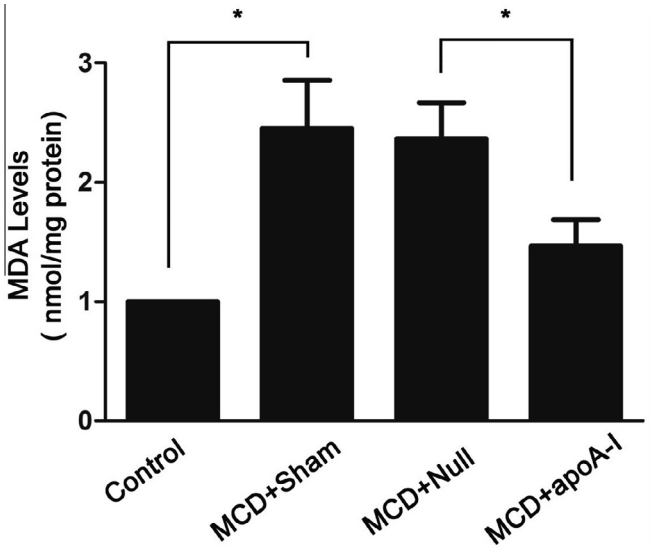

Fig. 3. ApoA-I overexpression alters the levels of lipid peroxidation in MCD diet-fed mice. Male C57BL/6J mice were divided into four groups and were fed chow or an MCD diet for 1 week with or without the injection of 1×10^8 pfu/ml of an empty adenoviral vector or a human apoA-I expressing vector into the femoral vein. MDA levels in the mouse livers were assayed using the OxiSelect TBARS assay kit. The results are representative of three independent experiments and are presented as the mean ± SEM. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

association between hepatic apoA-I, ROS, and COX-2, which strongly suggests that upregulation of apoA-I can effectively reduce hepatic steatosis by reducing ROS and inflammation.

Peroxidation is the primary mechanism by which polyunsaturated fatty acids generate ROS. Polyunsaturated fatty acids, including LA, can induce ROS production and promote AP-1 activity [22]. Steatohepatitis induced by an MCD diet has also been suggested to result from reactive oxygen species (ROS) [23], and ROS can act on accumulated fatty acids to form proinflammatory lipoperoxides [24]. As the rate-limiting step in reverse cholesterol transport, ABCA1 transports cellular cholesterol and phospholipids to apoA-I. This lipid transport process can reduce the levels of cellular lipids in hepatocytes, including free fatty acids [17]. This decrease of free fatty acids may result in decreased lipid peroxidation and ROS levels in these cells. This may explain why apoA-I overexpression had a beneficial effect on mice with MCD diet-induced NASH [17]. However, whether the anti-inflammatory effects of apoA-I extend beyond reverse cholesterol transport remains unclear; thus, apoA-I may also interact with other proteins to affect the signal transduction pathways related to inflammation.

MAPK controls the cellular responses to growth, apoptosis, and inflammation and can be activated in response to oxidative stress generation [25–27]. LPS induces the activation of MAPK through the induction of Nox-derived ROS, and the activation of MAPK is required for LPS-induced COX-2 expression in Hs68 cells [28]. MAPK can activate transcription factors such as AP-1 and NF-κB, which have been shown to regulate LPS-induced COX-2 expression [29]. Our results revealed that apoA-I activated MAPK and AP-1, and these results are consistent with the previous observation that eupafolin attenuated LPS-induced COX-2 expression by modulating the binding activity of AP-1 but not that of NF-κB [28].

This study has important therapeutic implications for the treatment of NASH. By modulating apoA-I expression, ROS levels and expression levels of the pro-inflammatory gene COX-2 were both significantly reduced. These effects may contribute to the removal of cholesterol and phospholipids. As illustrated in our previous study, overexpression of apoA-I may also suppress fatty acid synthesis through a decrease in LXR ligand levels [18]. As a result, the ABCA1/apoA-I pathway may function to decrease hepatocyte cellular lipid levels, which, in turn, causes decreased ROS and COX-2 expression levels and results in the antioxidant and anti-inflammatory effects of apoA-I in hepatocytes. Indeed, it has been suggested that dysfunction of apoA-I is connected to elevated ROS levels and steatosis [30]. Therefore, understanding the mechanisms of these processes would be useful for designing apoA-I-based therapeutic interventions that enhance the activity of hepatic lipid removal and prevent the development of NASH.

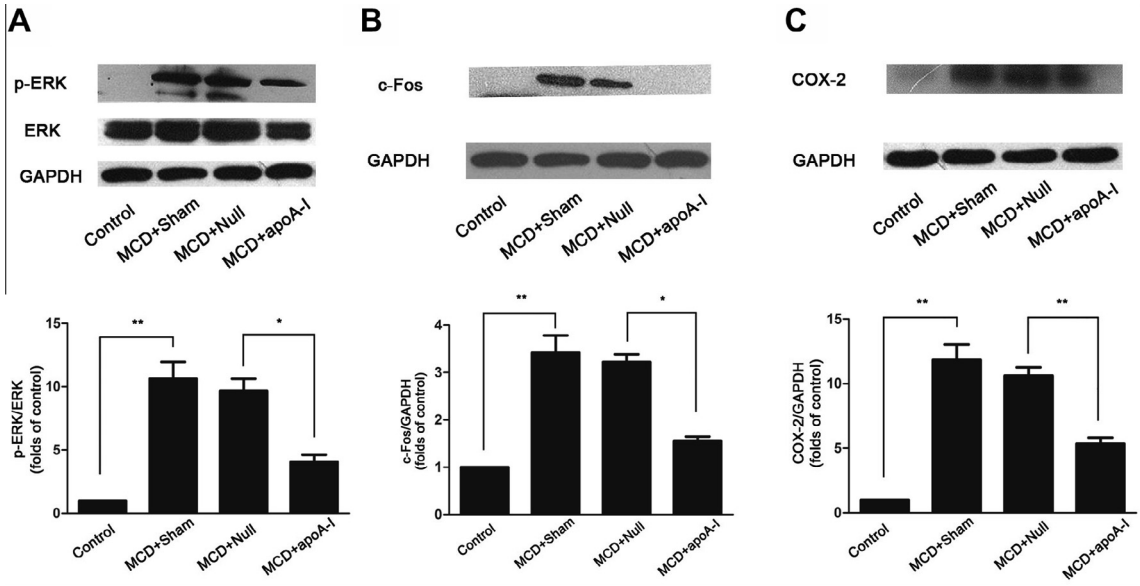

Fig. 4. ApoA-I overexpression decreases MAPK phosphorylation and c-Fos and COX-2 expression levels in MCD diet-fed mice. Male C57BL/6J mice were divided into four groups and were fed chow or an MCD diet for 1 week with or without the injection of 1×10^8 pfu/ml of an empty adenoviral vector or a human apoA-I expressing vector into the femoral vein. Hepatic proteins were isolated and analysed for p-ERK, ERK (A), c-Fos (B), and COX-2 (C) by Western blotting. The results are representative of three independent experiments and are presented as the mean ± SEM. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

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