Mipu1 Overexpression Protects Macrophages from oxLDL-Induced Foam Cell Formation and Cell Apoptosis

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Mipu1 (myocardial ischemic preconditioning upregulated protein 1) is a novel N-terminal Kruppel-associated box (KRAB)/C2H2 zinc finger superfamily protein, that displays a powerful effect in protecting H9c2 cells from oxidative stress-induced cell apoptosis. The present study aims to investigate the effect of Mipu1 overexpression on oxidized low-density lipoprotein (oxLDL)-induced foam cell formation, cell apoptosis, and its possible mechanisms. New Zealand healthy rabbits were used to establish atherosclerosis model, and serum levels of triglycerides, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol were detected by an automatic biochemical analyzer. Sudan IV staining was used to detect atherosclerotic lesions. The RAW264.7 macrophage cell line was selected as the experimental material. Oil red O staining, high-performance liquid chromatography, and DiI-labeled lipoprotein were used to detect cholesterol accumulation qualitatively and quantitatively, respectively. Flow cytometry was used to determine cell apoptosis. Real-time quantitative polymerase chain reaction (PCR) was used to detect the mRNA expression of the main proteins that are associated with the transport of cholesterol, such as ABCA1, ABCG1, SR-BI, and CD36. Western blot analysis was used to detect the protein expression of Mipu1. There were atherosclerotic lesions in the high-fat diet group with Sudan IV staining. High-fat diet decreased Mipu1 expression and increased CD36 expression significantly at the 10th week compared with standard-diet rabbits. Mipu1 overexpression decreased oxLDL-induced cholesterol accumulation, oxLDL uptake, cell apoptosis, and cleaved caspase-3. Mipu1 overexpression inhibited the oxLDL-induced CD36 mRNA and protein expression, but it did not significantly inhibit the mRNA expression of ABCA1, ABCG1, and SR-BI. Mipu1 overexpression inhibits oxLDL-induced foam cell formation and cell apoptosis. Mipu1 overexpression reduces the lipid intake of macrophages and might be associated with the downregulation of CD36 expression in the presence of oxLDL.

Introduction

THE MIGRATION OF CIRCULATING MONOCYTES into the subendothelial arterial space and their differentiation into macrophages are considered to be the essential steps of atherosclerosis (AS) development, which leads to rapid uptake of modified low-density lipoprotein (LDL) and subsequent foam cell formation. Foam cells are the characteristic pathological cells in atherosclerotic plaques and cholesterol accumulation is the most important reason for foam cell formation (Shashkin *et al.*, 2005; Das *et al.*, 2013). A variety of proteins are involved in cholesterol regulation. In particular, ATP-binding cassette transporters ABCA1 and ABCG1 play a pivotal role in cholesterol efflux from macrophage-derived foam cells, as they mediate the efflux of intracellular cholesterol and phospholipids to high-density lipoprotein (HDL) and apoA-I, respectively (Schmitz *et al.*, 2001; Feng and Tabas,

2002; Jessup *et al.*, 2006). In this way, cholesterol from peripheral cells is delivered to the liver for its conversion to bile acids (Rothblat *et al.*, 1999). Cluster of differentiation 36 (CD36) plays an important role in the uptake of oxidized low-density lipoprotein (oxLDL) (Rahaman *et al.*, 2006; Chen *et al.*, 2008), which is a well-known risk factor for AS, as the capacities of oxLDL uptake and foam cell formation are both decreased significantly in CD36^{-/-} mice (Febbraio *et al.*, 1999). SR-BI another scavenger receptor, mediates physiologically relevant, selective cholesterol transport, and plays a key role in controlling plasma lipoprotein and biliary cholesterol concentrations and reverse cholesterol transport (RCT). It has been proved that SR-BI can protect against early-onset AS in SR-BI/apolipoprotein E double homozygous knockout mice (Trigatti *et al.*, 1999; Connelly and Williams, 2004).

Mipu1 (myocardial ischemic preconditioning upregulated protein 1), a novel N-terminal Kruppel-associated

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box (KRAB)/C2H2 zinc finger superfamily protein, was identified because of its upregulation after myocardial ischemic preconditioning. It has an open reading frame of 1827 bp for encoding 608 amino acids with a KRAB domain and 14 C-terminal C2H2 zinc fingers (Yuan *et al.*, 2004; Jiang *et al.*, 2007). It has been proved that Mip1 can inhibit the apoptosis of H9c2 induced by H₂O₂ and TNF- α , and repress the expression of apoptosis-related genes Fas and Bax (Jiang *et al.*, 2007; Wang *et al.*, 2009). Recently, Wang *et al.* (2009) reported that hypoxia-inducible factor-1 α (HIF-1 α) bound to the hypoxia response element within the Mip1 promoter region and promoted its transcription, the cytoprotection of HIF-1 against H₂O₂-mediated injury in H9c2 cells partly through the regulation of Mip1 expression (Wang *et al.*, 2013). However, the role of Mip1 overexpression on oxLDL-induced lipid accumulation in macrophages has not been elucidated.

In this study, our findings indicate that a high-fat diet downregulated the expression of Mip1 in aortas of New Zealand healthy rabbits. Mip1 overexpression modulated lipid uptake and cell apoptosis induced by oxLDL through downregulating CD36 expression.

Materials and Methods

Animals and diets

New Zealand rabbits were obtained from the Experimental Animal Center of Hunan University of Traditional Chinese Medicine, China. The 24 rabbits were maintained under controlled temperature (21°C–23°C), 12-h light–12-h dark cycle and free access to food and water, no restrictions on male or female. All experiments were performed in the experimental animal department of the University of South China, Hengyang, China (certificate number: SCXK2010-0004) according to the institutional and government guidelines and approved by the local council of ethics. The rabbits were randomly divided into two groups: control group and high-fat diet group. The control group was fed with a standard diet, whereas the high-fat diet group was fed with a standard diet containing 2% cholesterol. All the rabbits were fed for 10 weeks.

Metabolic profile analysis

At the end of the 10th week, the rabbits were kept in fasting for at least 8 h before being anesthetized by 10% chloral hydrate, and blood samples were then collected from the ear edge vein. Serum centrifuged from the blood samples was used to measure levels of plasma lipids such as triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) using an automatic biochemical analyzer (Thermo Electron Co.). Aortas were also collected and quickly frozen in liquid nitrogen, and then stored at –80°C for later analysis.

Detection of atherosclerotic lesion area

For macroscopic analysis of plaque extension, the adventitia was removed from the aortas and fixed overnight in 10% paraformaldehyde. Then the fixed aortas were stained with Sudan IV, mounted, and then photographed by a digital camera.

Generation of stably transfected RAW264.7-vector and RAW264.7-Mip1 cells

The RAW264.7 macrophages were obtained from the Type Culture Collection of the Chinese Academy of Sciences. The RAW264.7 cells were transfected with pcDNA3.1-Mip1 or pcDNA3.1-vector (a kind gift of Dr. Xianzhong Xiao, Central South University, Changsha, China) using Lipofectamine[®] 2000 Transfection Reagent (Invitrogen), following the manufacturer's instructions. Stably transfected RAW264.7-vector and RAW264.7-Mip1 cells were selected in the culture medium, supplemented with 500 μ g/mL geneticin (G418; Invitrogen). RAW264.7 cells were maintained in DMEM (HyClone) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C under 5% CO₂.

Mip1 expression through western blot analysis

Aortic tissues were ground on ice with 300 μ L RIPA buffer per 20 mg, and then centrifuged at 10,000 g for 15 min to obtain the aortic protein. RAW264.7-vector and RAW264.7-Mip1 cells were washed in cold phosphate-buffered saline (PBS) and lysed in RIPA buffer for 30 min at 4°C. SDS-PAGE immunoblotting was used to quantify the expression of Mip1 (Jiang *et al.*, 2009) and GAPDH (Cell Signaling Technology). Electrophoresis and immunoblotting were employed as described previously (Qu *et al.*, 2010).

Lipids uptake assay

Three different methods were used to detect the uptake of lipids by macrophages. In the first assay, after treatment with oxLDL (all of the oxLDL were commercial, the TBARS content was ranging from 5 to 15 nmol MDA/mg cholesterol), the culture medium was removed, and cells were washed four times with PBS, and fixed with 4% paraformaldehyde for 20 min. Fixed cells were rinsed with PBS and stained with fresh Oil red O (Sigma) working solution (0.5% Oil red O in isopropanol:dH₂O 6:4) for 30 min. The nuclei were lightly stained with hematoxylin for 5 min. Stained cells were observed using an inverted microscope. For the second method, RAW264.7 cells were washed with PBS for three times after treatment with oxLDL. Protein content was determined by the BCA kit. Proteins were precipitated with 6% trichloroacetic acid, and neutral lipids were extracted with a mixture of hexane/isopropanol (v:v 4:1). The organic phase containing the lipids was collected in a vacuum freeze drying plant at 65°C and the residues were redissolved in 0.1 mL mixture of isopropanol/n-heptane/acetonitrile (v:v35:12:52). The mixture was centrifuged at 1500 rpm for 5 min. Cholesterol ester content was analyzed by high-performance liquid chromatography (Ma *et al.*, 2009). Briefly, the chromatographer (PerkinElmer, Inc.) system included a PerkinElmer series 600 LINK, a PerkinElmer series 200 vacuum degasser, a PerkinElmer series 200 UV/visible detector, a pump, and a reverse-phase column. Ten microliters of supernatants were injected into the column and eluted with isopropanol/n-heptane/acetonitrile (v:v, 35:12:52) at a flow rate of 1 mL/min. Absorbance at 216 nm was monitored. The products were identified by comparing the retention times and peak areas with authentic external standards. For the third method, oxLDL (v/v, 4:1) was labeled with Dil overnight at 37°C.

Seventy-five micrograms Dil-oxLDL was incubated with RAW264.7 cells for 24 h at 37°C; then cells were washed three times with PBS, and then lysed in lysis buffer. The protein concentration was detected by the BCA assay. Dil fluorescence was detected following excitation at 520 nm and emission at 590 nm using a 590 nm cut-off filter (Gemini XS microplate fluorometer). A fluorescence standard curve was prepared by diluting 0, 1, 2, 4, 8, 16, and 32 μ g Dil-oxLDL in lysis buffer. Results were normalized to total cell protein concentration.

Apoptosis detection by FACS

The apoptosis of RAW264.7 cells was detected by FACS analysis with the Annexin-V staining (BD Biosciences). RAW264.7 cells were incubated with 75 μ g/mL oxLDL for 24 h. The cells were then collected and incubated with PE-Annexin-V, cells were resuspended in PBS (2% paraformaldehyde), and detected by FACS. The percentage of PE-Annexin-V positive cells was quantified as the degree of apoptosis.

Caspase-3 activity assay

After incubation with 75 μ g/mL oxLDL for 24 h, cells were then lysed with passive lysis buffer (Promega) and the clear supernatant was used for caspase-3 activity assay after centrifugation according to the manufacturer's instructions. The protein concentration of the lysate was detected by the BCA kit and the relative caspase-3 activities were normalized to protein concentration.

Quantitative real-time reverse transcription PCR

Total RNA was extracted, and cDNA fragments were generated by reverse transcription. A total of 100 ng of cDNA was used in the real-time PCR to measure mRNA expression, whereas 10 ng of cDNA was used in the β -actin control PCR. The primer sequences used for the amplification were designed using the Primer 5.0 software and were evaluated using the Oligo 5.0 software. The primer sequences (mouse) for ABCG1 were: forward 5'-GGGTCTGAAGTCCCTACCT-3', reverse 5'-TACTCC CCTGATGCCACTTC-3' (NM_009593.2, 66 bp); for ABCA1: forward 5'-C CCCACTTTTTTCTTCCCTTTC-3', reverse 5'-GACACGAGACCGACGAG GGAG-3' (NM_013454.3, 128 bp); for CD36: forward 5'-GAACCTATTGAAGGCTT ACA TCC-3, reverse 5'-CCCCA GTCACCTTGTTTGAAC-3' (NM_001159557.1, 246 bp); for SR-BI: forward 5'-TTTGGA GTGGTAGTAAAAAG GGC-3', reverse 5'-TGACATCAG GGACTCAGAGTAG-3' (NM_001205083.1, 72 bp); for β -actin: forward 5'-TGGCCGGGACCTGACAGACTA-3', reverse 5'-ATCTGAAGCTCGTC CTCTACCGG-3' (NM_007393.3, 142 bp). SYBR Green I fluorescence dye (Takara Biotechnology) was used to bind specifically to the minor groove of double-stranded DNA. All reactions followed the typical sigmoidal reaction profile, and cycle threshold was used as a measurement of amplicon abundance as described previously (Zhu *et al.*, 2011).

Statistical analyses

The results are expressed as mean $\pm$ SEM. Statistical analyses were performed with the Graphpad Prism V.5 soft-

ware using the Student's *t*-test. Significance was taken as $p < 0.05$.

Results

Change of Mipu1 and CD36 protein expression in thoracic aorta

Circulating levels of TC, TG, and LDL-C were remarkably increased in the high-fat diet group of New Zealand rabbits relative to those in the standard-diet fed group, and no difference of serum HDL-C concentration was observed between these two groups $p > 0.05$ (Fig. 1A). Gross examination of the aorta revealed atherosclerotic lesion in the high-fat diet group with Sudan IV staining (Fig. 1B). As shown in Figure 1C, the high-fat diet decreased Mipu1 and increased CD36 expression significantly at the 10th week compared with the standard-diet rabbits $p < 0.05$ (Fig. 1D).

Mipu1 overexpression inhibits oxLDL-induced lipid accumulation in macrophages

We overexpressed pcDNA3.1-Mipu1 in RAW264.7 cells (Fig. 2A), and used pcDNA3.1 as a control. Since uptake of oxLDL is the event that triggers the formation of lipid-laden foam cells, we examined the effect of Mipu1 overexpression on lipid accumulation in macrophages. The Oil red O staining (Fig. 2B) and intracellular TC quantitative assay (Fig. 2C) indicated that macrophage lipid content in the presence of oxLDL was significantly decreased in cells transfected with pcDNA3.1-Mipu1 compared with those transfected with pcDNA3.1. To further investigate the role of Mipu1 overexpression in lipid accumulation in RAW264.7 cells, we detected the effect of Mipu1 overexpression on oxLDL uptake by macrophages. As shown in Figure 2D, Mipu1 overexpression inhibited the uptake of oxLDL in macrophages. These data suggested that Mipu1 overexpression inhibited the oxLDL-induced lipid accumulation in macrophages.

Mipu1 overexpression attenuated oxLDL-induced apoptosis

Overexpression of Mipu1 significantly attenuated oxLDL-induced RAW264.7 cell apoptosis (PE-Annexin-V positive) ($p < 0.05$, $n = 4$) (Fig. 3A). Mipu1 overexpression also significantly reduced caspase-3 activities (Fig. 3B).

Mipu1 overexpression reduced CD36 expression in the presence of oxLDL

It is known that many key genes are involved in the efflux and uptake of cholesterol by macrophages, and we determined whether Mipu1 overexpression modulates mRNA expression of ABCA1, ABCG1, SR-BI, and CD36 by real-time PCR in RAW264.7 cells. The mRNA levels of ABCA1, ABCG1, SR-BI, and CD36 were similar in RAW264.7-vector versus RAW264.7-Mipu1 cells in the untreated group (Fig. 4). Compared with the control group, the CD36 mRNA/protein expression and ABCA1 mRNA expression were both increased in the oxLDL-treated group ($p < 0.05$), whereas the mRNA expression of SRB1 and ABCG1 showed no significant change. Mipu1 overexpression inhibited the oxLDL-induced expression of CD36 mRNA and protein, whereas the expression of ABCA1 mRNA showed no significant change.

FIG. 1. Change of Mip1 (myocardial ischemic preconditioning upregulated protein 1) and CD36 expression in high-fat diet induced atherosclerosis in rabbits. **(A)** Plasma levels of total triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) after 10 weeks of high-fat diet feeding. **(B)** Plaque quantification of thoracic aorta stained with Sudan IV. Protein expression of Mip1 **(C)** and CD36 **(D)** in the aortic tissue ($n=4$ each), values are mean $\pm$ SEM, * $p < 0.05$ versus control group.

Discussion

There is evidence showed that hyperglycemia and hypertriglyceridemia have direct effects on the arterial wall and induced endothelial dysfunction (Arcaro *et al.*, 2002). The elevation of TC, TG, and LDL-C was observed in high-fat diet rabbits in our study (Fig. 1A), and there were atherosclerotic lesions in the high-fat diet group and the Mip1 expression in aortic tissues was much lower in the high-fat diet group (Fig. 1B, C). At the same time, we and others (Yao *et al.*, 2014) demonstrated that CD36 expression in aortic tissues was increased significantly in the high-fat diet group. It has been suggested that CD36 plays an important role in foam cell formation and progression of AS. However, the precise mechanism underlying oxLDL-induced CD36 upregulation has not been completely clarified. Our

results in the present study suggested that the capacity of Mip1 in participating in the process of lipid accumulation might be related to regulation of CD36 expression.

Foam cell formation is a hallmark of the early stages of AS and plays an important role in plaque progression and instability (Fogelman *et al.*, 1980; Boyle *et al.*, 2012). Increasing evidences have indicated that the foam cells found in the atherosclerotic reaction are macrophages that are derived from blood-borne monocytes or smooth muscle cells. The hallmark of these foam cells is that the cholesterol ester content is over 50% of the total intracellular cholesterol (Fogelman *et al.*, 1980; Boyle *et al.*, 2012). RAW264.7 macrophages are easy to culture and have strong phagocytic and adhesive abilities, so it has been widely used in studies of the pathways of foam cell formation (Kuchibhotla *et al.*, 2008; Steinbusch *et al.*, 2011).

FIG. 2. Mipu1 overexpression inhibits oxLDL-induced lipid accumulation in macrophages. **(A)** LDH release in oxidized low-density lipoprotein (oxLDL)-treated group (75 $\mu\text{g/mL}$ of oxLDL for 24 h) was $23.4\% \pm 3.1\%$ (relative to total LDH release), and control group (no oxLDL treatment) was $5.0\% \pm 0.7\%$. **(B)** Expression of Mipu1 protein in RAW264.7 cells transfected with pcDNA3.1-Mipu1 was analyzed by western blot. **(C)** RAW264.7-vector and RAW264.7-Mipu1 cells were treated with 75 $\mu\text{g/mL}$ oxLDL for 24 h, and the extent of lipid loading was assessed by Oil red O staining. **(D)** Under the same conditions as in **(C)**, the intracellular cholesterol content was measured through high-performance liquid chromatography. **(E)** Dil-oxLDL fluorescence intensity in cells incubated with Dil-labeled oxLDL (75 $\mu\text{g/mL}$) for 24 h. Data are presented as the mean $\pm$ SEM of at least four independent experiments. # $p < 0.05$ versus control group; * $p < 0.05$ versus pcDNA3.1 group.

FIG. 3. Mipu1 overexpression inhibited apoptosis of RAW264.7 cells. RAW264.7-vector and RAW264.7-Mipu1 cells were treated with 75 $\mu\text{g/mL}$ oxLDL for 24 h. **(A)** FACS analysis with PE-Annexin-V staining revealed that Mipu1 overexpression inhibited apoptosis. **(B)** Caspase-3 activities were measured as described in methods. Data are presented as the mean $\pm$ SEM of at least four independent experiments. * $p < 0.05$ versus pcDNA3.1 group.

FIG. 4. Mip1 overexpression inhibited CD36 expression in the presence of oxLDL. RAW264.7-vector and RAW264.7-Mip1 cells were incubated with 75 μ g/mL oxLDL for 24 h and the amount of mRNA encoding ABCA1, ABCG1, SR-BI, and CD36 was measured by quantitative PCR (**A–D**) (data show mean fold change relative to untreated RAW264.7-vector $\pm$ SEM from four independent experiments). (**E**) Protein expression of CD36 was detected by western blot after oxLDL treatment. [#] $p < 0.05$ versus untreated group, ^{\$} $p < 0.05$ versus RAW264.7-vector group.

In this study, macrophages meet the typical characteristic of foam cells after treatment with 75 μ g/mL oxLDL for 24 h. To determine the role of Mip1 overexpression in foam cell formation, a RAW264.7 cell line, stably expressing Mip1, was generated. With the use of this cell line, we detected the change of intracellular cholesterol. In this study, we showed that Mip1 overexpression not only decreased uptake of oxLDL, but also synergistically reduced the oxLDL-induced intracellular cholesterol accumulation and inhibited foam cell formation. Until now, no study has reported the correlation between Mip1 and AS. Our study is the first to discover that Mip1 overexpression inhibited the formation of foam cells.

Both smooth muscle cells and macrophages undergo apoptosis in atherosclerotic plaques (Kockx and Herman, 2000). Macrophage cell apoptosis promotes the development of the necrotic core, which is a key factor in rendering plaques vulnerable to disruption and acute luminal thrombosis (Seimon and Tabas, 2009). Caspase-3 is a key protein in apoptotic pathways (Hutter *et al.*, 2004). Our study proved that overexpression of Mip1 inhibited the oxLDL-induced RAW264.7 cell apoptosis, as well as the amount of activated caspase-3.

Assessment of foam cell formation evidenced an additional, new role for Mip1 overexpression by enhancing macrophage lipid storage. Increased cell cholesterol accumulation could be a result of an increased uptake, decreased

efflux, or both. Therefore, the efflux of cholesterol was the next factor evaluated as a potential mechanism involved in the macrophage accumulation of lipids induced by Mipu1. Cholesterol efflux from macrophages is mainly directed by members of the ABC transporter family, such as ABCG1 and ABCA1, through active RCT, in a process that requires the presence of apoA-I or HDL as cholesterol acceptors (Neculai *et al.*, 2013; Fu *et al.*, 2013; Daniil *et al.*, 2013). Real-time PCR experiments indicated that the mRNA levels of the transporter ABCG1 and ABCA1 were not changed significantly in Mipu1-expressing RAW264.7 cells in the presence of oxLDL. Thus, overall, Mipu1 does not seem to play a major role in cholesterol efflux. The uptake of oxLDL leading to focal lipid accumulation in foam cells is intimately involved in the formation of early fatty streak lesions, as well as in the evolution of more complex atherosclerotic plaques (Ashraf and Gupta, 2011; Hutter *et al.*, 2004; Amezaga *et al.*, 2014). In fact, our findings suggest that decreased foam cell formation by Mipu1 overexpression is mainly a result of decreased lipid uptake. Extensive evidence points to a significant role of CD36, the major macrophage scavenger receptor responsible for oxLDL, in the progression of AS and suggests it could be an important target for therapeutic treatment (Febbraio *et al.*, 2000; Kuchibhotla *et al.*, 2008; Ashraf and Gupta, 2011; Steinbusch *et al.*, 2011; Neculai *et al.*, 2013), so we hypothesized that Mipu1 overexpression may be decreasing oxLDL uptake by modulating CD36 expression and/or activity. Indeed, Mipu1 expression induced the downregulation of CD36 mRNA/protein levels in RAW264.7-Mipu1 cells versus RAW264.7-vector cells upon oxLDL treatment. These data suggested that Mipu1 overexpression may inhibit the oxLDL-induced lipid accumulation in macrophages and might be associated with modulation of CD36 expression. It is proposed that there are also other mechanisms in addition to the decrease of CD36 expression that are responsible for the inhibition of lipid accumulation by Mipu1 overexpression.

In summary, our results support the notion that Mipu1 is an anti-atherogenic protein that participates in multiple events of macrophage homeostasis, including macrophage survival, oxLDL uptake, and foam cell formation, which might be associated with the downregulation of CD36 expression. Our findings are of relevance for the understanding of Mipu1 that contribute to the prevention of AS.

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Disclosure Statement

No competing financial interests exist.

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