Int J Clin Exp Pathol 2015;8(9):10625-10634
www.ijcep.com /ISSN:1936-2625/IJCEP0012107

Original Article

MCPIP is induced by cholesterol and participated in cholesterol-caused DNA damage in HUVEC

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Received June 29, 2015; Accepted July 29, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Hypercholesterolemia is an important risk factor for atherosclerosis and cholesterol treatment would cause multiple damages, including DNA damage, on endothelial cells. In this work, we have used human umbilical vein endothelial cell line (HUVEC) to explore the mechanism of cholesterol induced damage. We have found that cholesterol treatment on HUVEC could induce the expression of MCPIP1. When given 12.5 mg/L cholesterol on HUVEC, the expression of MCPIP1 starts to increase since 4 hr after treatment and at 24 hr after treatment it could reach to 10 fold of base line level. We hypothesis this induction of MCPIP1 may contribute to the damaging process and we have used siRNA of MCPIP1 in further research. This MCPIP1 siRNA (siMCPIP) could down regulate MCPIP1 by 73.4% and when using this siRNA on HUVECs, we could see the cholesterol induced DNA damage have been reduced. We have detected DNA damage by γH2AX foci formation in nuclear, γH2AX protein level and COMET assay. Compare to cholesterol alone group, siMCPIP group shows much less γH2AX foci formation in nuclear after cholesterol treatment, less γH2AX protein level in cell and also less tail moment detected in COMET assay. We have also seen that using siMCPIP1 could result in less reactive oxygen species (ROS) in cell after cholesterol treatment. We have also seen that using siMCPIP could reduce the protein level of Nox4 and $p47^{phox}$, two major regulators in ROS production. These results suggest that MCPIP1 may play an important role in cholesterol induced damage.

Keywords: MCPIP1, siMCPIP, HUVEC, cholesterol, DNA damage, ROS

Introduction

Atherosclerosis is a chronic disease of vessel walls and has serious and extensive impact on human health. It is now a leading cause of death throughout the world [1]. The formation of atherosclerotic lesions starts with endothelial cell damage, lipid deposition and vascular smooth muscle cell proliferation [2, 3]. Hypercholesterolemia is considered to be an important risk factor for the occurrence of atherosclerosis. *In vitro* and *in vivo* epidemiological experiments have revealed that a large amount of cholesterol esters are deposited in cells of artery wall and intercellular of atherosclerosis, which is induced in experimental animal with high saturated fat and high-cholesterol diet and also can be found in patients [4, 5]. The mechanism of how hypercholesterolemia leads to atherosclerosis is not clear yet and researches on how plasma lipoprotein-derived cholesterol would affect structure and function of vessel wall will help in understanding this procession.

In recent year, the relationship between atherosclerosis and DNA damage is aroused as a new perspective in the study of atherosclerosis development. Nuclear DNA (nDNA) damage and lack of mitochondria DNA (mtDNA) copies are detected in the circulating blood cells and plaque cells of patients with coronary heart disease [6]. DNA adducts are found in atherosclerosis plaque cells [7] and atherosclerosis plaque tissue also shows the presence of DNA microsatellite instability with higher risk of losing heterozygosity in DNA than normal tissue [8]. One of the main factors causing DNA damage is oxidative stress. Long-term exposure to hyperlipidemia, smoking and diabetes, especially in the case of metabolic abnormalities increases the production of reactive oxygen

species (ROS), induces cellular antioxidant capacity imbalances and causes oxidative stress [9]. Our previous works have found that the free cholesterol could lead to elevated intracellular ROS, resulting in genomic DNA damage in human umbilical vein endothelial cell line (HUVEC) [10, 11]. These results suggest that cholesterol metabolic abnormalities may lead to hypercholesterolemia deposited in artery wall and contribute to atherosclerosis. It still needs further work to clarify the molecular mechanism of how hypercholesterolemia leads to DNA damage.

MCPIP (monocyte chemoattractant protein-1 induced protein, MCPIP), discovered in 2006, is a novel zinc finger transcription factor with pro-apoptotic activity induced by MCP-1. Till now, four members of MCPIP have been reported, named as MCPIP1 (ZC3H12a, located on chromosome 1p34.3), MCPIP2 (ZC3H12b, Xq12), MCPIP3 (ZC3H12c, 1q22.3) and MCPIP4 (ZC3H12d, 6q25.1) [12]. Currently, most of studies are focusing on MCPIP1. In the study of cardiac myoblasts death [13], adipocytes generate [14] and osteoclast differentiation experiments [15], MCPIP1 is found to activate NADPH oxidase, induce ROS overproduction and endoplasmic reticulum stress and trigger autophagy [16]. In our previous works, we have found that DNA damage induced by free cholesterol in the HUVEC was caused by increased oxidative stress, and the cellular ROS was mainly from NADPH oxidase [10, 11]. Thus, we hypothesize that MCPIP1 might be an important factor in mediating DNA damaging process via increasing ROS in HUVECs.

Materials and methods

Cell culture and identification

HUVEC was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell was maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 8% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (both from Invitrogen, MA) and incubated at 37°C in a humidified atmosphere with 5% CO_2/95% air. Unless otherwise indicated, experiments with endothelial cells were performed on early passage cultures. HUVEC was identified by immunofluorescence. Briefly, cell was grown on glass cover slips and fixed with 100% acetone at -20°C for 15 min, then detecting the factor VIII-related antigen (VIII-Rag) by a polyclonal antibody for VIII-Rag (BOSTER, CA). A goat anti-rabbit IgG with Cy3 fluorescein-labeled secondary antibody was used and fluorescent was visualized with an Olympus IX71 fluorescence microscope.

RNA interference treatment

HUVECs were seeded into 24 well plates at 1~2.5 × 10^4 cells per well and cultured overnight to get cell fusion rate of 30%~50%. Transient transfection were performed using 25 nM siRNA targeting MCPIP1 or 25 nM non-specific siRNA (Both from Thermo, MA) as the negative control. SiRNA was mixed with 0.5~2.5 μl Dharmafect transfection agent (Dharmacon Research, CO) according to the manufacturer's guide. Transfection efficiency was monitored by Real-time PCR and Western blotting. All transfection were performed in triplicate and repeated at least three times.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from treated HUVECs using TRIzol (Invitrogen) following the manufacturer's instruction. RNA amount was determined by the optical density (OD) value of RNA samples and integrity of RNA was determined by denatured electrophoreses on 1% agarose-formaldehyde gels. 1 μg total RNA was used for cDNA synthesize using cDNA synthesized kit (Takara, JAP) following manufacturer's guide. Primers designed for real-time PCR were: MCPIP F-5'-gtggtcatcgatgggagcaa-3' and R-5'-cctccaggatggcacaaaca-3' and β -actin primers (Takara) were used as internal control. Quantitative real-time PCR was performed with iCycler Thermal Cycler (Bio-Rad, CA) using 2 × SYBR® *Premix Ex Taq*™ (TakaRa). Forty cycles were conducted as follows: 95°C for 30 s, 58°C for 30 s, proceeded by 1 min at 72°C for polymerase activation. Quantification was performed by the delta cycle time method, and normalized to β-actin. Results presented are of three independent experiments, each determined from triplicate loaded wells.

Western blotting analysis

HUVECs (1 × 10^6 cells) were washed with ice-cold phosphate buffered saline (PBS) and scrapped of the dish into 100 μL lysis buffer

containing 1% sodium dodecylsulphate, 1 mM phenylmethylsulphonyl fluoride, Protease Inhibitor Cocktail (Beyotime, CHN), and 25 mM Tris-HCl, pH 6.8. The resultant lysates were centrifuged at 12000 rpm for 10 min at 4°C to remove particulate material. For $p47^{phox}$ activation assay, membrane proteins were prepared using gradient ultracentrifugation. Total protein contents were measured by bicinchoninic acid (BCA, from Thermo) method following the user's guide. 40 μg total protein sample mixed with 5 × SDS loading buffer were denatured by boiling for 10 min, then separated by 10% polyacrylamide gel electrophoresis under reducing conditions and transferred to PVDF (Millipore, MA). Samples were then subjected to immune-blotting using rabbit polyclonal antibodies specific for γ-H2AX (1:1000), NOX4 (1:200), $p47^{phox}$ (1:200, These three antibodies are from Santa Cruz Biotechnology, CA) MCPIP1 (1:200), and β-actin (1:2500, these two antibodies are from Abcam, MA). Horseradish peroxidase-labelled anti-rabbit IgGs (Beyotime) secondary antibody was used for all experiments at 1:5000 dilution. Immunoreactive bands were detected using enhanced chemoluminescence kit (ECL, from Thermo). The relative intensity of the bands was quantized by scanning densitometry using the Image J software.

Oxidative stress detection

ROS production was determined by total intracellular oxidant in cultured HUVECs, which was measured 24 hrs after treatment with cholesterol using 10 μmol/L 2'-7'-dichlorofluorescein diacetate (DCHF-DA, from Sigma, MO) for 30 min at 37°C in water bath and were analyses by flowcytometry (Beckman Coulter Ltd, UK). The excitation wave length is 488 nm and emission is 530 nm. Measurements were carried out for 3 samples in each group independent. Results were compared using the median fluorescent intensity of the whole cell population.

DNA damage determination

DNA damage was analyzed via three different ways: (1) COMET assay: DNA damage was quantified using single cell gel eletrophoresis (SCGE), also known as COMET Assay, kit (KeyGEN Biotech) according to the manufacturer's instruction. The COMET slides were stained with propidium iodide (PI) and analyzed by fluorescent microscope. 100 cells were evaluated in each sample and 3 slides per treatment were analyzed. Data was analyses using the COMET Assay Software Project (CASP software). DNA damage was quantified by measuring the tail moment (TM) calculated as percentage of DNA in the tail × tail length. (2) γH2AX foci formation assay: Immunostaining was performed on cells grown on glass cover slips and fixed with 4% paraformaldehyde solution at 4°C for 15 min, then permeablized in 0.2% Triton X-100 on ice for 15 min. Foci of γ-H2AX were detected with a monoclonal antibody for H2AX histone (Upstate) and FITC-conjugated goat anti-mouse antibody. Nuclear counterstaining with DAPI was performed after removal of excess secondary antibody. Immunostaining was visualized with an Olympus IX71 fluorescence microscope. (3) Western blot analysis of γ-H2AX was performed as previously described.

Statistical analysis

Data from at least three independent experiments were used for statistical analysis. The experimental data was analyzed by using SPSS statistical software (SPSS Inc.) under windows 8. All values are presented as means ± SD. Multiple group comparison was performed by one-way ANOVA followed by the Turkey's HSD for comparison of means. Comparisons between two groups were performed using Student t tests. Values of $P<0.05$ were considered to be statistically significant.

Results

Cholesterol induces MCPIP expression in HUVECs

MCPIP1 is an inducible gene and some inflammatory cytokines, such as IL-1β and TNF-α, can induce the expression of MCPIP in endothelial cells or smooth muscle cells. However, no one has reported the link between cholesterol treatment and expression of MCPIP1 yet. In this work, we firstly observed the induction of MCPIP1 in HUVECs by cholesterol. In this experiment, HUVECs were administrated with different dose of cholesterol from 3.13 mg/L, 6.25 mg/L, 12.5 mg/L, 25 mg/L to 50 mg/L for 24 hr treatment. A serum-free RPMI-1640 plus ethanol group was used as control group. After treatment, cells were harvested for Real-time PCR analysis and the results showed that MCPIP1 mRNA expression level was increased

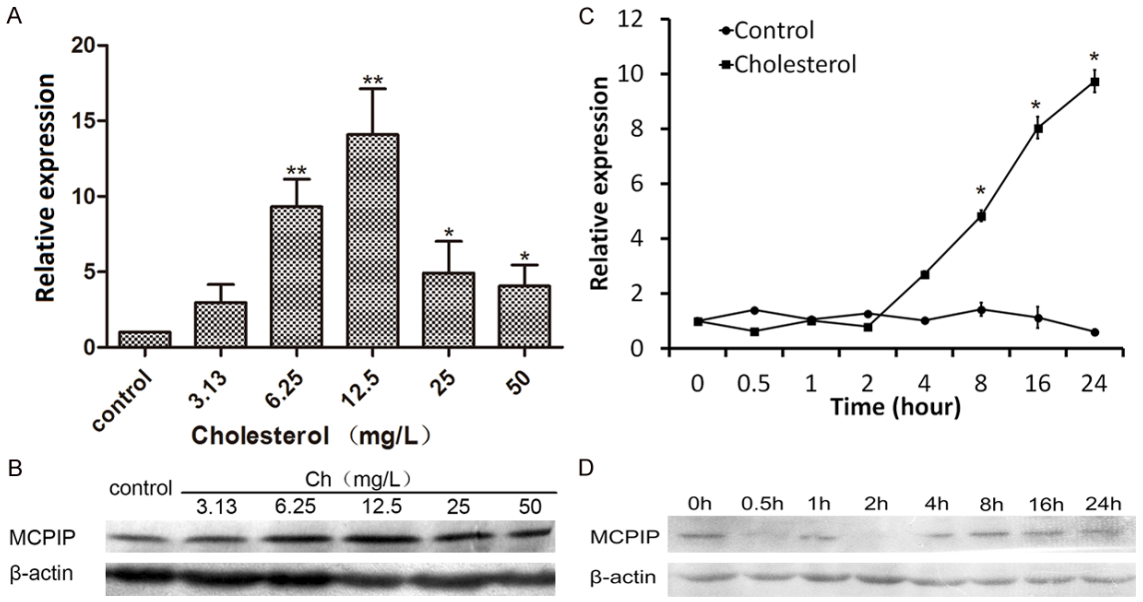

Figure 1. Cholesterol induces MCPIP expression in HUVECs. A. Induction of MCPIP1 by different dose of cholesterol after 24 hr treatment, detected by real-time PCR assay. Results were shown as relative amount normalized to control group. B. Western blotting assay of MCPIP1 protein level in total protein extract from HUVECs treated with different dose of cholesterol for 48 hr. *: $P<0.05$, **: $P<0.01$ vs. control. C. Time cause response of MCPIP expression in HUVECs treated by 12.5 mg/L cholesterol for different duration (5 min, 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h). Determined by Real-time PCR assay and the results were shown as relative amount normalized to control group. *: $P<0.01$ vs. control. D. Time cause response of MCPIP protein level determined by western blotting assay.

after cholesterol treatment (**Figure 1A**). When using cholesterol from 3.13 mg/L to 12.5 mg/L, the amount of MCPIP1 is increasing along with increasing concentration of cholesterol, and peaked at 12.5 mg/L with more than 12-fold induction compare to control group. In 25 mg/L or 50 mg/L group, the expression of MCPIP1 is lower than 12.5 mg/L but still significantly higher than control. To confirm the real-time PCR result, we have also detected MCPIP1 protein in HUVECs by Western blot. As shown in **Figure 1B**, the MCPIP1 protein level is increased after cholesterol treatment and 12.5 mg/L group shows the strongest MCPIP1 signal, which is consistent with the real-time PCR result.

In the next step, we examined the MCPIP1 expression in HUVECs at different time points (5 min, 0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h and 24 h) after stimulation of 12.5 mg/L cholesterol. Real-time PCR assay result (**Figure 1C**) shows that in the first 4 hr after cholesterol administration, there's no induction of MCPIP1 in HUVECs. From 4 hr after cholesterol administration, MCPIP1's mRNA level starts to increase and 24 hr shows the highest MCPIP1 level (**Figure 1C**). To validate this result, we have detected of MCPIP protein expression in HUVECs with 12.5 mg/L cholesterol at different times by western blotting (**Figure 1D**) and it also shows that protein of MCPIP1 is increased after 8hr cholesterol treatment. According to the relationship of dose-response and time cause response, we conclude that the optimal condition for cholesterol to induce MICPI1 on HUVECs is 12.5 m/L cholesterol for 24 hours. Take together; we have observed that cholesterol treatment could induce expression of MCPIP1 in HUVECs and increases MCPIP1 protein level in cell.

MCPIP1 is participated in cholesterol-induced DNA damage process

We have reported that cholesterol treatment could induce DNA damage in HUVECs before. When we observed that expression of MCPIP1 was up regulated in cholesterol treatment, we hypothesized that MCPIP1 might be an important regulator in the DNA damaging process. To explore whether MCPIP1 was involved in DNA damage process, we had used interference RNA to silence MCPIP1 and then determine the

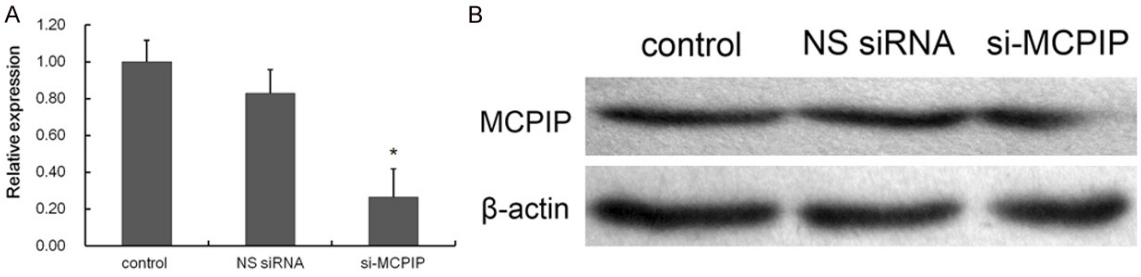

Figure 2. Silence of MICPI1 by siRNA in HUVECs. A. Real-time PCR assay on HUVECs transfected with MCPIP1 specific siRNA (siMIPIP1) or non-targeting siRNA (NS siRNA). Results were shown as relative amount normalized to control group. *: *P*<0.05 vs. Ns siRNA. B. Western blotting assay on HUVECs transfected with siMCPIP1 or NS siRNA. The silence efficiency of MCPIP was calculated by (1-siMCPIP/control)*100%.

DNA damage induced by cholesterol in HUVECs. After optimization of transiently transfection on HUVECs, we had found that the best condition for HUVECs was using 25 nM siMIPIP with 1 μl Dharmafect transfection agent and transfect for 24 hr (data not shown). To evaluate the effect of MCPIP1 siRNA, we had used real-time PCR to determine mRNA level of MCPIP1 and Western blot to determine protein level after siMIPIP transfection. As shown in **Figure 2A**, 24 hr after transfection, the siMCPIP group shows significant lower amount of MCPIP1 mRNA while NS siRNA group shows no difference to control group. The silent efficiency of MCPIP mRNA is 73.4% compare to control group. The protein level of MCPIP1 in HUVECs is detected 48 hr after treansfection and as shown in **Figure 2B**, it is much lower in the siMCPIP group than NS siRNA or control group. These results indicate that using MCPIP1 siRNA could effectively decrease MCPIP1 mRNA amount and protein level in HUVECs.

We then used siMCPIP transfected HUVECs in cholesterol treatment. HUVECs were transfected with siMCPIP or NS siRNA for 24 hr, then were treated by 12.5 mg/L cholesterol group (CH) or ethanol as control group. To determine the DNA damage caused by cholesterol treatment, we had used three ways. The first method we've used detecting γH2AX foci formation in nuclear, which is a widely used assay for detecting DNA double-strand breaks [18]. After cholesterol treatment, HUVECs were fixed and stained by anti-γH2AX antibody to show the foci and nuclear was stain by DAPI. The result images were show in **Figure 3A**. We have also used Image-pro plus 6.0 software to quantify the percentage of positive cells in each group and also to get the frequency of γH2AX foci in each positive cell and the result is shown in **Figure 3B**. After 24 hr cholesterol treatment, most HUVECs would show positive γH2AX foci staining in nuclear while when the MCPIP1 was silent by siMCPIP, the γH2AX foci positive cell was much less than cholesterol group and the frequency in nuclear was also lower. The decrease is significant in both percentage of positive cell and frequency of foci in nuclear. (in both cases, *P*<0.01 compare to cholesterol group).

The second method we have used to determine DNA damage was analysis γH2AX protein level in HUVECs by Western blotting analysis. As shown in **Figure 3C**, the γH2AX protein level was increased in cholesterol group, while in HUVECs that transfected with siMCPIP, the γH2AX protein level is not much lower the cholesterol group. This result is very similar as the result of γH2AX foci formation assay and suggests that down regulation of MCPIP1 would lead to less DNA damage induced by cholesterol.

The third way we have used to show DNA damage after cholesterol treatment was COMET assay. After siRNA transfection and 12.5 mg/L cholesterol 24 hr treatment, we have detected the individual cells' DNA damage by COMET assay and used CASP software measured each cell's tail moment (TM) after DNA electrophoresis, namely, DNA content of cells and the DNA migration distance (length of comet tail). As shown in **Figure 3C** and **3D**, the result shows the DNA damage in HUVECs treated with 12.5 mg/L cholesterol as indicated by much longer comet tail and less amount of DNA in cell body. Like the results of γH2AX associate assays, with transfection of siMCPIP, the cell could

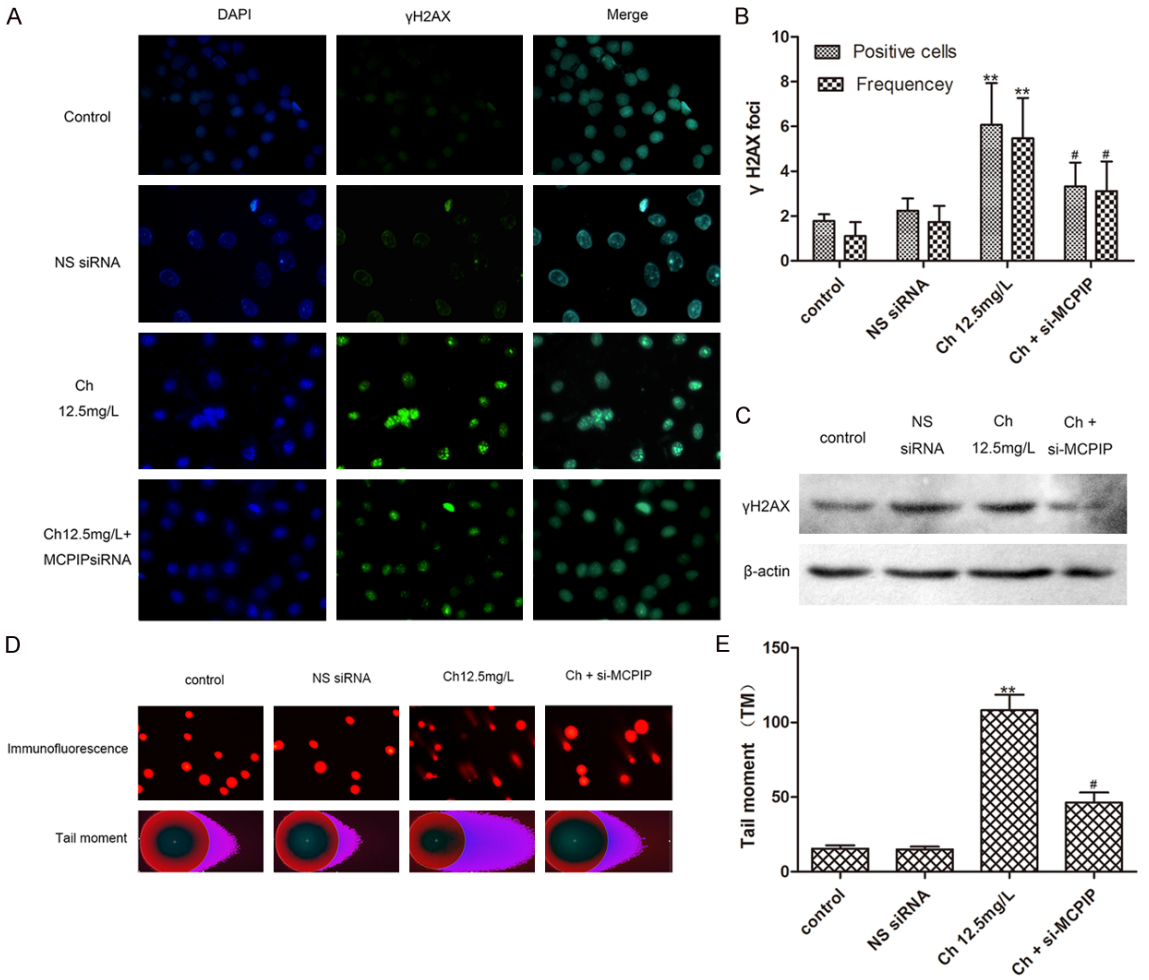

Figure 3. Measurement of cholesterol-induced DNA damage after the silence of MCPIP in HUVECs. A. Immunofluorescent staining on HUVECs tranfected with siMCPIP1 or NS siRNA for 24 hr, then treated by 12.5 mg/L cholesterol for 24 hr. Staining of γH2AX is green color to show foci formation and nuclear is stained in blue color by DAPI. B. Image-pro plus 6.0 software analyzed the positive cell ratio (the percentage of positive cells in the total number of cells in each group) and frequency (the ratio of the number of γH2AX foci in total cell number) of γH2AX foci formation. *: $P<0.05$ vs. cholesterol group, **: $P<0.05$ vs. control group. C. Western blotting analysis for determine γH2AX protein level in HUVECs. D. Detecting individual cells' DNA damage by COMET assay after cholesterol treatment. E. CASP software measured single cell's tail moment (TM) after DNA electrophoresis. **: $P<0.01$ vs. control group, #: $P<0.05$ vs. cholesterol group.

show less DNA damage as indicated by a shorter comet tail and higher amount of nuclear DNA than cholesterol group. After evaluation of the comet TM value, the result indicates that in siMCPIP group, the comet TM value is decreased by half of the value in CH group and the difference is significant ($P<0.01$).

Overall, these results show that after silence of MCPIP1, HUVECs could suffer less DNA damage from cholesterol treatment. This finding suggests that MCPIP1 was involved in the DNA damaging process in cholesterol treatment. To understand the mechanism on how MCPIP1 mediated the cholesterol-induced DNA damage, we have performed further in-depth exploration.

MCPIP1 increases the production of ROS, increases Nox4 expression and increases membrane translocation of p47^{phox} in cholesterol treated HUVECs

MCPIP is a factor which is closely related with the development of inflammation response in ischemic heart disease, diabetes heart disease and obesity. Mechanism studies reveal that increase of ROS production, a downstream

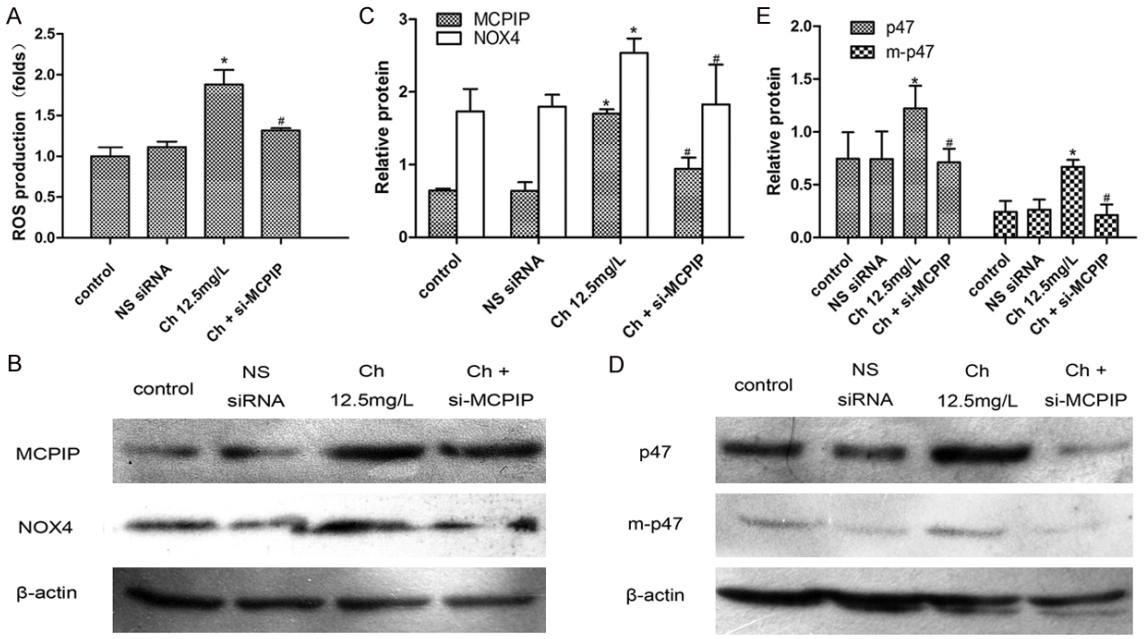

Figure 4. MCPIP mediates the production of ROS, Nox4 expression and translocation of $p47^{phox}$ onto the membrane in cholesterol-treated HUVECs. A. Intracellular ROS determined by 2',7'-dichlorofluorescein diacetate (DCFDA) and analyses by flow cytometry. B&C. Determine expression of Nox4 by Western blotting assay. D&E, Determine expression of total $p47^{phox}$ and membrane $p47^{phox}$ by Western blotting assay. *: $P<0.05$ vs. control group, #: $P<0.05$ vs. cholesterol group.

event after induction of MCPIP, plays an essential role on the endoplasmic reticulum stress. The increase of ROS production by induction of MCPIP might be the key factor to introduce DNA damage after cholesterol treatment in HUVECs. In this experiment, we tried to determine whether ROS production is increased after cell exposed to cholesterol. We used 2',7'-dichlorofluorescein diacetate (DCFDA) as a probe to measure ROS in HUVECs. As shown in **Figure 4A**, cells in cholesterol group displayed an increased production of ROS than control group. After administration of siMCPIP to knock down MCPIP1 in HUVECs, the production of ROS was significant lower than cholesterol group. This finding suggests that increase of ROS production by MCPIP1 might be the factor to induce DNA damage and knock down of MCPIP1 would reduce ROS production and thus lead to less DNA damage. This result is in agreement with our previous study, which have found that cells treated an antioxidant, n-acetylcysteine (NCA), together with cholesterol could show a decrease in γH2AX accumulation [10, 11] and indicates that increased ROS production is a key step in cholesterol triggered DNA damage, while MCPIP1 is a major regulator in ROS production.

To understand how MCPIP1 could increase ROS production in HUVECs, we have analysis two of the factors which are important for ROS production. Nox4, a homologous protein of cytoplasm subunit $gp91^{phox}$ of phagocyte NADPH oxidase, is a major enzyme in endothelial cell to produce ROS. Another important event in ROS production is the activation and translocation of cytoplasm subunit $p47^{phox}$ into membrane to boost ROS generation. In this experiment we have used Western blotting analysis on both Nox4 and $p47^{phox}$ after cholesterol treatment and knock down of MCPIP1 in HUVECs. The results showed that cholesterol treatment increased the expression of Nox4, increased total $p47^{phox}$ and membrane $p47^{phox}$ protein level in HUVECS. Moreover, when knock down MCPIP1 by siMCPIP, the Nox4, total $p47^{phox}$ and membrane $p47^{phox}$ were much lower than cholesterol group. These results indicate that increased MCPIP expression may induce expression of Nox4 and increase membrane translocation of $p47^{phox}$, both of which lead to more ROS production (**Figure 4B-E**).

Discussion

In this study, we have used different dose of cholesterol to treat HUVECs and detected induction of MCPIP1 by qPCR and Western blot. The results show that using 12.5 mg/L cholesterol 24 hr treatment on HUVECs can significant increase MCPIP1's mRNA level and protein level in cell. In a time cause experiment, we have found that induction of MCPIP1 starts from 4 hr after cholesterol treatment and keep increasing till 24 hr. The induction of MCPIP1 by cholesterol has not been reported yet and establishment of this link would expand the sight on understanding how cholesterol exerts its impact on endothelial cells.

In recent research, it has been found that any kind of stimulus-induced DNA double-strand breaks (DSBs) would lead to phosphorylation of H2AX (gamma-H2AX, γH2AX) and form the cluster of γH2AX foci [18]. Further experiments demonstrated that the formation of γH2AX foci could be served as a standard in detecting DSBs [19, 20]. In this study, we had also used γH2AX as the marker for detecting DNA damage in HUVECs. We had used both immunostaining and Western blotting to detect γH2AX level in HUVECs. The results show that after cholesterol treatment, γH2AX protein level was increased in HUVECs and γH2AX staining showed more foci formation in nuclear. These results serve a very strong indication of DNA damage induced by cholesterol. Moreover, we have observed that when using siMCPIP to knock down MCPIP1 in HUVECs, it shows much less γH2AX foci formation and lower γH2AX protein levels in compare to cholesterol alone group. These γH2AX results indicate that decrease of MCPIP1 protein level reduces DNA damage caused by cholesterol treatment and suggest that MCPIP1 was an important factor in DNA damaging process induced by cholesterol.

These years, DNA damage in cell could be visualized by single cell gel electrophoresis, an *in-situ*, sensitive and fast fluorescence method for detecting DNA damage in individual cells. In normal cell, nuclear DNA is well packaged by histone and could only show very limited tail like structure, while a damaged DNA would be migrated by the electric force and show a long tail like structure after fluorescent staining of DNA [21]. We have also used this assay to directly show DNA damage in HUVEC cell after cholesterol treatment. The results show that after cholesterol treatment, HUVEC cells have significant reduced cell body DNA and a much longer tail compare to control group. This result directly shows the DNA damage after cholesterol treatment. After knock down MCPIP1, the cell could show less DNA damage in cholesterol treatment by increased nuclear staining and shorter in tail length compare to cholesterol alone group. The changes of γH2AX and TM in HUVECs before and after MCPIP1 silencing indicated that MCPIP is an important factor in cholesterol- induced DNA damage in HUVECs.

To further understand how MCPIP1 is participated in DNA damage process, we have checked downstream events in MCPIP1 signal. Our previous work has indicated that cholesterol treatment can lead to elevation in intracellular ROS [10]. The tight link between accumulation of ROS and DNA damage urged us to explore whether MCPIP1 was associated with formation of ROS. The results show that by administration of siMCPIP, cell could indeed display a lower level of ROS than that in cholesterol group. It suggests that MCPIP1 might be an important molecular for ROS formation in HUVECs. As functional research on MCPIP1 is mainly a transcriptional factor, MCPIP1 must be activating one or more ROS producing enzymes to induce ROS in cell. In this work, we have picked two major ROS producing enzyme and analyses their level after using cholesterol and siMCPIP. One of the enzyme is NADPH oxidase, which is constituted by the cytoplasmic enzyme subunit $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and small molecules GTP-binding protein (Rac) and membrane subunits $gp91^{phox}$ and $p22^{phox}$ [22]. The other one is Nox4, which is also an indispensable enzyme of ROS in endothelial cells. Nox4 could constitute a functional complex binding with $p22^{phox}$ and this heterodimer, Nox-$p22^{phox}$, could be translocated on the membrane of endothelial cell. The phosphorylated $p47^{phox}$ subunit activates the Nox-$p22^{phox}$ via member translocation and anchors it [23, 24]. In order to explore the status of ROS producers, we had examined Nox4, total $p47^{phox}$ and membrane-$p47^{phox}$ level. Immunoblot analysis showed that cholesterol increased the expression of Nox4, total $p47^{phox}$ and membrane $p47^{phox}$ and then inhibited by MCPIP siRNA. These results indicated that MCPIP1 could elevate intracellular ROS by regulating Nox4 and $p47^{phox}$ membrane

translocation and activating ROS producing activity.

In this work, we have revealed the relationship among cholesterol, MCPIP1, ROS and DNA damage. When endothelial cells were exposed to cholesterol, expression of MCPIP1 would be significant induced. Induction of MCPIP1 could further up regulating Nox4 and NADPH oxidase and increasing membrane bonding $p47^{phox}$ thus increase ROS productionin cell. The increased ROS finally results in DNA damage. When we using siMCPIP to specifically silence MCPIP1, we have observed that the cholesterol induced DNA damage is decreased. The decreased DNA damage is surely associated with the fact that siMCPIP could also reduce ROS in cell and reduce the induction of Nox4 and $p47^{phox}$ by cholesterol. This finding confirms that MCPIP1 is a key factor in cholesterol induced DNA damage and further research on the mechanism would be important to prevent cell damage caused by cholesterol.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (30760078).

Disclosure of conflict of interest

None.

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