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Metformin reduces lipid accumulation in macrophages by inhibiting FOXO1-mediated transcription of fatty acid-binding protein 4

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ABSTRACT

Objective: The accumulation of lipids in macrophages contributes to the development of atherosclerosis. Strategies to reduce lipid accumulation in macrophages may have therapeutic potential for preventing and treating atherosclerosis and cardiovascular complications. The antidiabetic drug metformin has been reported to reduce lipid accumulation in adipocytes. In this study, we examined the effects of metformin on lipid accumulation in macrophages and investigated the mechanisms involved. *Methods and results:* We observed that metformin significantly reduced palmitic acid (PA)-induced intracellular lipid accumulation in macrophages. Metformin promoted the expression of carnitine palmitoyltransferase I (CPT-1), while reduced the expression of fatty acid-binding protein 4 (FABP4) which was involved in PA-induced lipid accumulation. Quantitative real-time PCR showed that metformin regulates FABP4 expression at the transcriptional level. We identified forkhead transcription factor FOXO1 as a positive regulator of FABP4 expression. Inhibiting FOXO1 expression with FOXO1 siRNA significantly reduced basal and PA-induced FABP4 expression. Overexpression of wild-type FOXO1 and constitutively active FOXO1 significantly increased FABP4 expression, whereas dominant negative FOXO1 dramatically decreased FABP4 expression. Metformin reduced FABP4 expression by promoting FOXO1 nuclear exclusion and subsequently inhibiting its activity. *Conclusions:* Taken together, these results suggest that metformin reduces lipid accumulation in macrophages by repressing FOXO1-mediated FABP4 transcription. Thus, metformin may have a protective effect against lipid accumulation in macrophages and may serve as a therapeutic agent for preventing and treating atherosclerosis in metabolic syndrome.

Introduction

The accumulation of lipids in macrophages is a hallmark of atherosclerotic lesion formation that not only contributes to cholesterol and triglyceride retention within the vascular wall, but also increases vascular oxidative stress and inflammation [1]. Developing strategies to lower lipid level and reduce lipid accumulation in macrophages may have therapeutic potential for preventing and treating atherosclerosis and cardiovascular complications.

Metformin, the first-line therapy for managing type 2 diabetes, has been reported to inhibit triglyceride and cholesterol synthesis and lower VLDL and LDL levels in patients with diabetes [2–4]. However, whether it can directly reduce lipid accumulation in macrophages has not been examined. Metformin has been shown to decrease intracellular lipid levels and improve insulin sensitivity in pre-adipocytes, hepatocytes and skeletal muscle [5–7]. It is conceivable that it may also directly reduce lipid accumulation in macrophages and thus prevent the formation of atherosclerosis.

In the present study, we examined the effects of metformin on palmitic acid (PA)-induced lipid accumulation in macrophages and investigated the molecular mechanisms involved. We observed that metformin significantly reduced PA-induced intracellular lipid accumulation in macrophages. Metformin prevented lipid accumulation in macrophages by inhibiting the FOXO1-mediated transcription of fatty acid-binding protein 4 (FABP4). Thus, metformin may have the potential to prevent atherosclerosis formation.

Materials and methods

Cell culture. The human monocytic leukemia cell line THP-1 (ATCC, Manassas, VA) was cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% L-glutamine. Differentiation of THP-1 cells into macrophages was induced by 100 nM phorbol 12-myristate 13-acetate (PMA) (Alexis Biochemical, Farmingdale, NY) for 72 h. Then, the macrophages were transfected with siRNA or plasmid DNA and/or treated with PA or metformin at various concentrations for the various time periods indicated in the text.

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Transfection of cells with siRNA and plasmid DNA. FABP4 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and FOXO1 siRNA was purchased from Dharmacon (Chicago, IL). Silencer® Negative control siRNA (Ambion, Austin, TX) was used as a negative control. Various FOXO1 plasmid DNAs (Addgene, Cambridge, MA) were used in this study. Transfection of THP-1 macrophages with siRNA or plasmid DNA was performed with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the method described [8]. Transfected cells were then treated with fatty acid or metformin at the concentrations for the time periods indicated in the text. The efficiency of transfection was determined by Western blot.

Western blot analysis. THP-1 macrophages were washed twice with PBS and whole cell extracts were prepared as described [9]. Protein concentrations were measured by using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Cell lysates were subjected to SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked, incubated overnight with primary antibody, washed, and then incubated with secondary horseradish peroxidase-labeled antibody. Antigen detection was performed with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. The data shown were representative of 3 separate experiments. FABP4 and carnitine palmitoyltransferase I (CPT-1) antibodies were purchased from Santa Cruz Biotechnology. Protein expression was quantified by densitometry by using Quantity One software (Bio-Rad). Relative protein levels were normalized to β-actin and expressed as percentages of the control.

Quantitative real-time PCR (Q-PCR). Q-PCR was conducted as described [10]. Total RNA from treated cells was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. Total cellular mRNA was reverse-transcribed into cDNA by using the iScript cDNA synthesis kit (Bio-Rad). Q-PCR was performed by using the iCycler iQ real-time PCR detection system (Bio-Rad). Primers were designed with the use of Beacon Designer 2.0 software. The following primers for human FABP4 were used: forward, 5′-ATGAT AAACTGGTGGTGGAAT-3′; reverse, 5′-ATCAGCTTGGGAGAAAATT AC-3′. The mRNA levels were acquired from the value of the threshold cycle (Ct) of the target gene normalized to the Ct of β-actin. The data shown represent 3 separate experiments.

Detection of intracellular lipid accumulation. Intracellular lipid accumulation was determined by oil red O staining as described previously [11]. THP-1 macrophages were transfected with siRNA and/or treated with PA in the presence or absence of metformin for 24 h and washed with PBS. Treated cells were fixed with 4% formaldehyde for 90 min and incubated with oil red O working solution for 30 min. Absorbance at 490 nm was measured. Staining intensity was measured and normalized to cell number. The mean staining intensity was calculated from 3 independent experiments. Intracellular lipid levels were compared with the no-treatment control and expressed as percentages of the control.

Statistical analysis. All quantitative variables are presented as means ± SD from 3 separate experiments. The difference between two groups was assessed with the use of an independent *t*-test. We compared the differences of 3 or more groups with a one-way ANOVA. A two-tailed *P*-value of $P < 0.05$ was considered statistically significant.

Results

Metformin reduced fatty acid-induced lipid accumulation in THP-1 macrophages

We first tested whether metformin could reduce lipid accumulation in macrophages, which can be significantly induced by free fatty acids (unpublished data). Macrophages differentiated from human

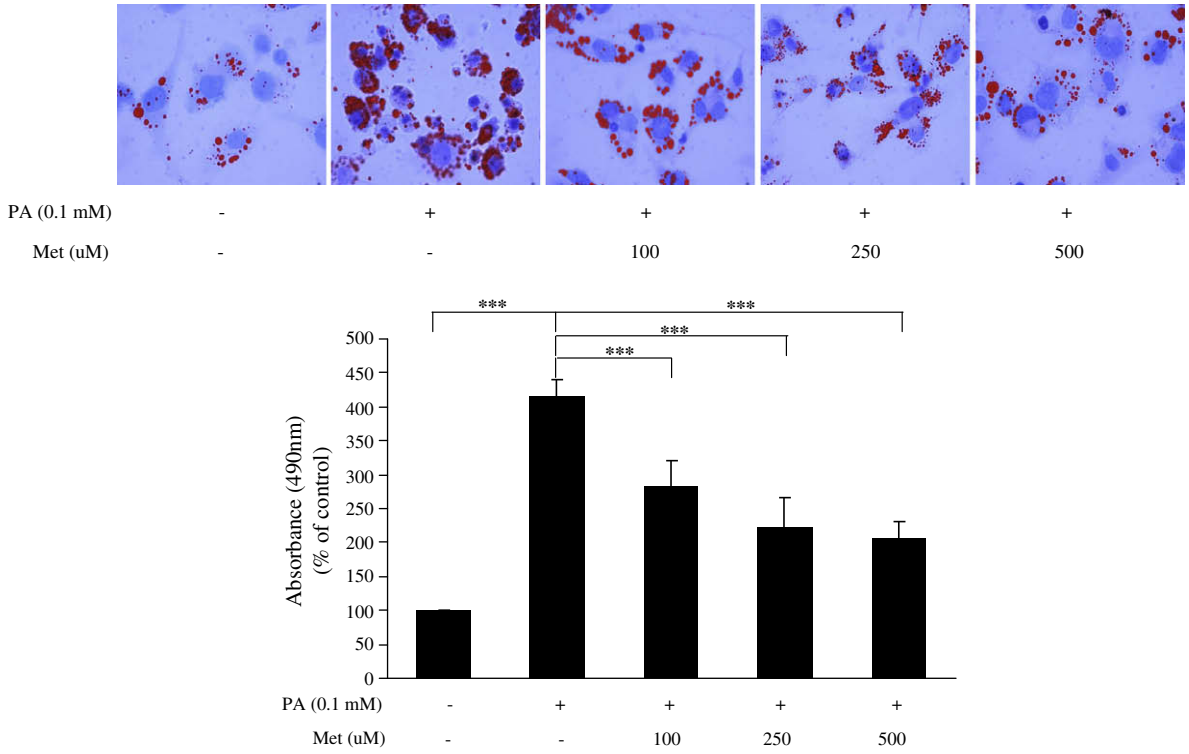

Fig. 1. Metformin significantly reduced the PA-induced increase of intracellular lipid accumulation in human THP-1 macrophages. THP-1 macrophages were treated with increasing amounts of metformin in the presence of PA. Intracellular lipid accumulation was assessed by oil red O staining. Staining intensity was measured and normalized to cell number. The mean staining intensity was calculated for 3 randomly selected fields per coverslip. Intracellular lipid levels were compared with the no-treatment control and expressed as percentages of the control. Representative images and quantitative analysis of oil red O staining from 3 independent experiments are shown. Data represent as means ± SD. *** $P < 0.001$ vs PA treatment alone.

THP-1 cells were incubated with increasing amounts of metformin in the presence of PA, and lipid accumulation was examined by oil red O staining. As shown in Fig. 1, PA significantly increased intracellular lipid accumulation, an observation consistent with previous report [12]. Importantly, the PA-induced increase in intracellular lipid accumulation was reduced by metformin in a dose-dependent manner, indicating that metformin is capable of reducing intracellular lipid accumulation in macrophages.

Metformin reduced lipid accumulation in macrophages by downregulating FABP4 and upregulating CPT-1

We next investigated the mechanisms for the metformin-induced reduction of lipid accumulation observed in macrophages. Lipid accumulation is controlled by a few key molecules, such as FABP4, a fatty acid transport protein involved in fatty acid uptake and CPT-1, a rate-limiting enzyme involved in fatty acid oxidation. We assessed the effect of metformin on the expression of these molecules. As shown in Fig. 2A, metformin alone slightly decreased FABP4 expression and increased CPT-1 expression. In the presence of PA, metformin significantly decreased PA-induced FABP4 expression, and increased CPT-1 expression in a dose-dependent manner (Fig. 2B). Importantly, the knockdown of FABP4 by siRNA decreased basal intracellular lipid accumulation and prevented PA-induced intracellular lipid accumulation (Fig. 2C), suggesting a critical role for FABP4 in macrophage lipid accumulation. We therefore, focused our study on FABP4 regulation.

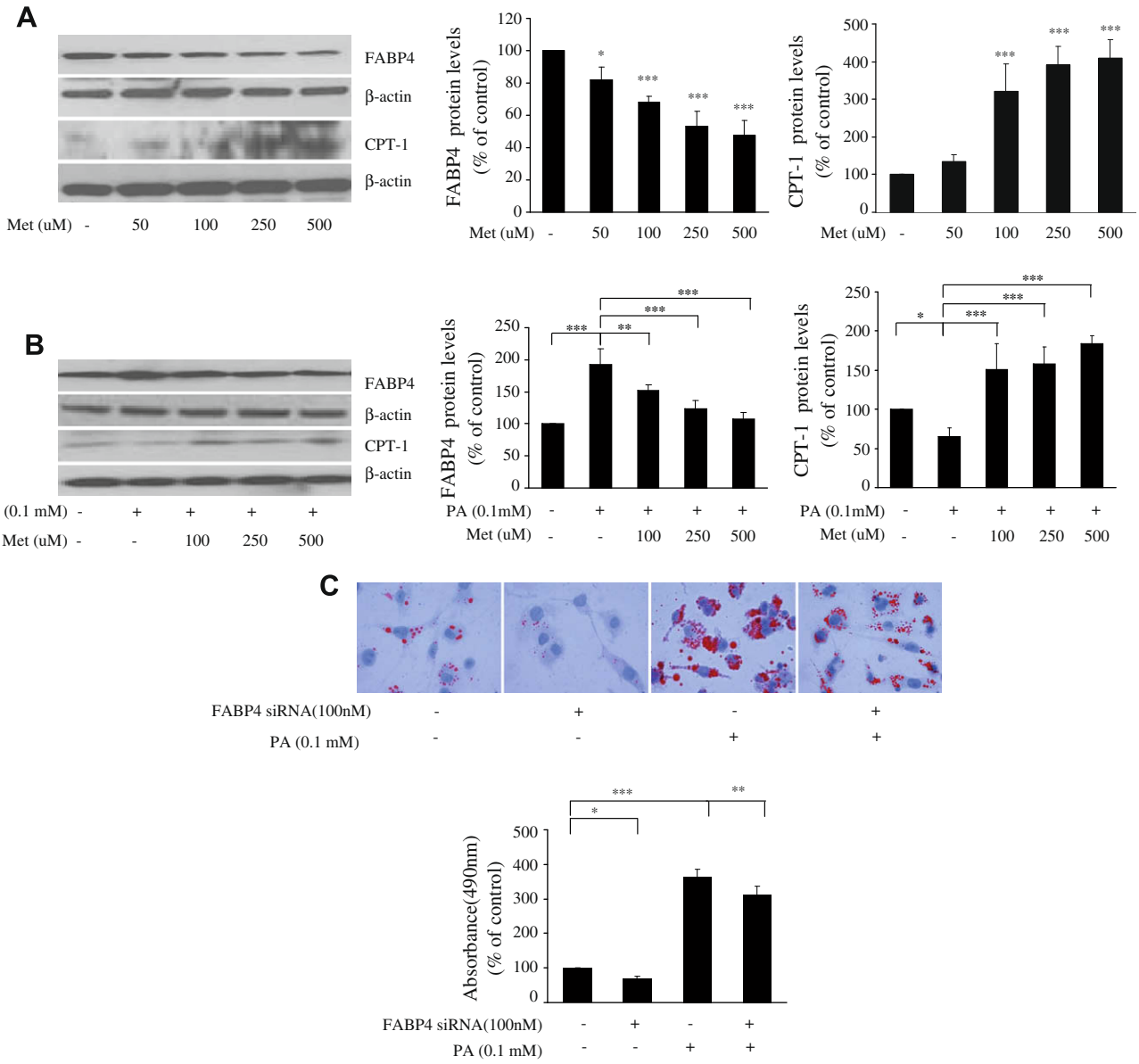

Fig. 2. Metformin reduced lipid accumulation by downregulating FABP4. (A, B) Metformin inhibited FABP4 and increased CPT-1 expression in the absence or the presence of PA. Human THP-1 macrophages were treated with increasing amounts of metformin in the absence (A) or presence (B) of PA. FABP4 and CPT-1 expression was examined by Western blot. The relative levels of protein were compared and expressed as percentages of the control. Representative blots and quantitative analyses from 3 independent experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the no-treatment control, or as indicated. (C) Involvement of FABP4 in PA-induced intracellular lipid accumulation. Human THP-1 macrophages were transfected with FABP4 siRNA and then treated with PA for 24 h. Intracellular lipid accumulations were detected with oil red O staining, and staining intensity was measured and normalized to cell number. Intracellular lipid levels were compared with the no-treatment control and expressed as the percentage of the control. Representative staining and quantitative analysis are shown. Data represent the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Metformin regulated FABP4 expression at the mRNA level

To further investigate the mechanisms of metformin-induced FABP4 repression, we examined whether metformin affected the expression of FABP4 mRNA. We found that PA increased FABP4 mRNA levels, which were significantly reduced by metformin in a dose-dependent manner (Fig. 3). Indicating that metformin may decrease FABP4 expression at the mRNA level either by decreasing transcription or by promoting mRNA degradation.

Metformin reduced FABP4 expression by inhibiting FOXO1

To investigate the mechanisms by which metformin induces the downregulation of FABP4 mRNA, we examined the transcriptional regulation of FABP4. Transcription factor FOXO1 has been shown to regulate lipid metabolism. We therefore, examined whether FOXO1 can regulate FABP4 expression in macrophages. As shown in Fig. 4A, inhibiting FOXO1 expression with FOXO1 siRNA significantly reduced basal and PA-induced FABP4 expression suggesting that FOXO1 mediates the PA-induced upregulation of FABP4 expression. Additionally, the overexpression of wild-type FOXO1 (FOXO1 WT) and constitutively active FOXO1 (FOXO1 CA) significantly increased FABP4 expression, whereas dominant negative FOXO1 (FOXO1 DN) dramatically decreased FABP4 expression (Fig. 4B) indicating that FOXO1 positively regulates FABP4. Importantly, treating cells with metformin significantly reversed the FOXO1-induced induction of FABP4 expression supporting the notion that metformin may downregulate FABP4 expression by inhibiting FOXO1. Indeed, immunostaining showed that PA increased FOXO1 nuclear translocation (Fig. 4C). Metformin not only maintained FOXO1 in the cytoplasm, but also prevented PA-induced FOXO1 nuclear translocation, indicating that metformin can effectively inhibit FOXO1 nuclear translocation. Together, these data suggest that metformin may inhibit FABP4 expression by inhibiting FOXO1 nuclear translocation and subsequently affecting its target transcription.

Discussion

In the present study, we show that metformin reduced fatty acid-induced lipid accumulation in macrophages. Metformin reduced lipid accumulation in macrophages by inhibiting FOXO1-mediated FABP4 transcription. A proposed mechanism for the metformin-mediated reduction of intracellular lipid accumulation is shown (Fig. 4D).

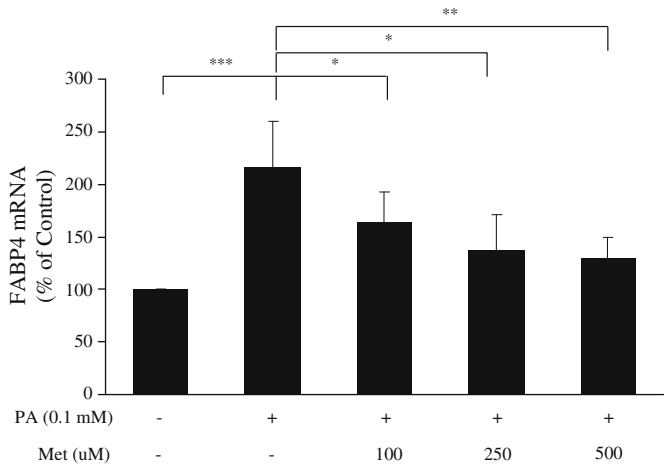

Fig. 3. Metformin regulates FABP4 expression at the mRNA level. Human THP-1 macrophages were treated with metformin in the presence of PA for 24 h. FABP4 mRNA levels were examined by Q-PCR and normalized with β-actin mRNA. The relative levels of mRNA were compared and expressed as percentages of the control. Data represent the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs PA treatment. Metformin induced a significant dose-dependent decrease in FABP4 mRNA.

Free fatty acids are biologic molecules that can be used as metabolic fuels at physiologic concentrations. However, when fatty acids are oversupplied (as seen in metabolic syndrome), lipid accumulation increases in macrophages (Song et al., unpublished data), thereby facilitating inflammation and apoptosis that contribute to the formation and progression of atherosclerosis.

Metformin has profound beneficial effects in metabolism and reduce insulin resistance. However, the protective effects of metformin on human atherosclerosis are less clear [13–15], even though it has been reported to reduce the development of atherosclerotic lesions in animal models [16]. Studies have shown that metformin can correct dyslipidemia in humans [2–4]. In this study, we show that metformin can directly reduce lipid accumulation in macrophages, suggesting a potential role of metformin in reducing foam cells and preventing atherosclerosis. Metformin has been shown, to reduce the expression of pro-inflammatory cytokines [17], ameliorate oxidative stress [18,19] and protect endothelial cell function [20]. With all these beneficial effects on lipid lowering, macrophage lipid reduction and vascular protection [21–23], metformin may have the potential to prevent the development of atherosclerotic lesions. However, further studies are necessary to test this hypothesis *in vivo*.

We further investigated the mechanisms involved in the metformin-induced reduction of intracellular lipid accumulation. Reduction of intravascular lipid accumulation can be achieved by preventing fatty acid uptake, inhibiting triglyceride synthesis, and promoting lipolysis and fatty acid-beta oxidation. Metformin has been shown to reduce lipid accumulation by increasing fatty acid oxidation [5–7]. Here, our results show that metformin can decrease fatty acid-induced upregulation of FABP4—a novel mechanism that explains the effect of metformin on reducing intracellular lipid accumulation. FABP4, a member of the cytosolic 14- to 15-kDa FABP family, is ubiquitously expressed in macrophages and adipocytes and is involved in fatty acid transport and metabolism [24–26]. It has been shown to promote intracellular lipid accumulation and inflammation [27,28]. Lack of FABP4 expression significantly reduces lipid accumulation and inflammation in macrophages and protects hyperlipidemic mice from developing atherosclerotic lesions [28,29], supporting a critical role for FABP4 in atherosclerosis development. Thus, downregulation of FABP4 by metformin may be an important mechanism for preventing excessive lipid accumulation in macrophages and vascular inflammation.

Our study shows that activation of metformin reduced FABP4 expression by inhibiting FOXO1, which we identified as a transcription factor that positively regulates FABP4 expression and mediates PA-induced FABP4 induction. FOXO transcription factors play important roles in regulating lipid metabolism [30]. FOXO1 has also been shown to induce lipolysis in adipocyte [31] and stimulate the production of VLDL [32] in hepatocytes. In muscle cells, FOXO1 has been shown to promote fatty acid uptake and enhance fatty acid oxidation, with fatty acid uptake exceeding oxidation and net triglyceride accumulation [33]. Elevated FOXO1 expression and nuclear localization have been observed in mouse models of diabetes and obesity, and inhibition of FOXO1 has been shown to reduce hepatic fatty accumulation and improve insulin sensitivity [34,35]. In this study, we show for the first time that FOXO1 positively regulates the transcription of FABP4, suggesting FOXO1 may play an important role in regulating fatty acid uptake and lipid accumulation in macrophages, thus contributing to the formation of atherosclerotic lesions. However, future *in vivo* studies are required to examine the role of FOXO1 in the formation and progres-

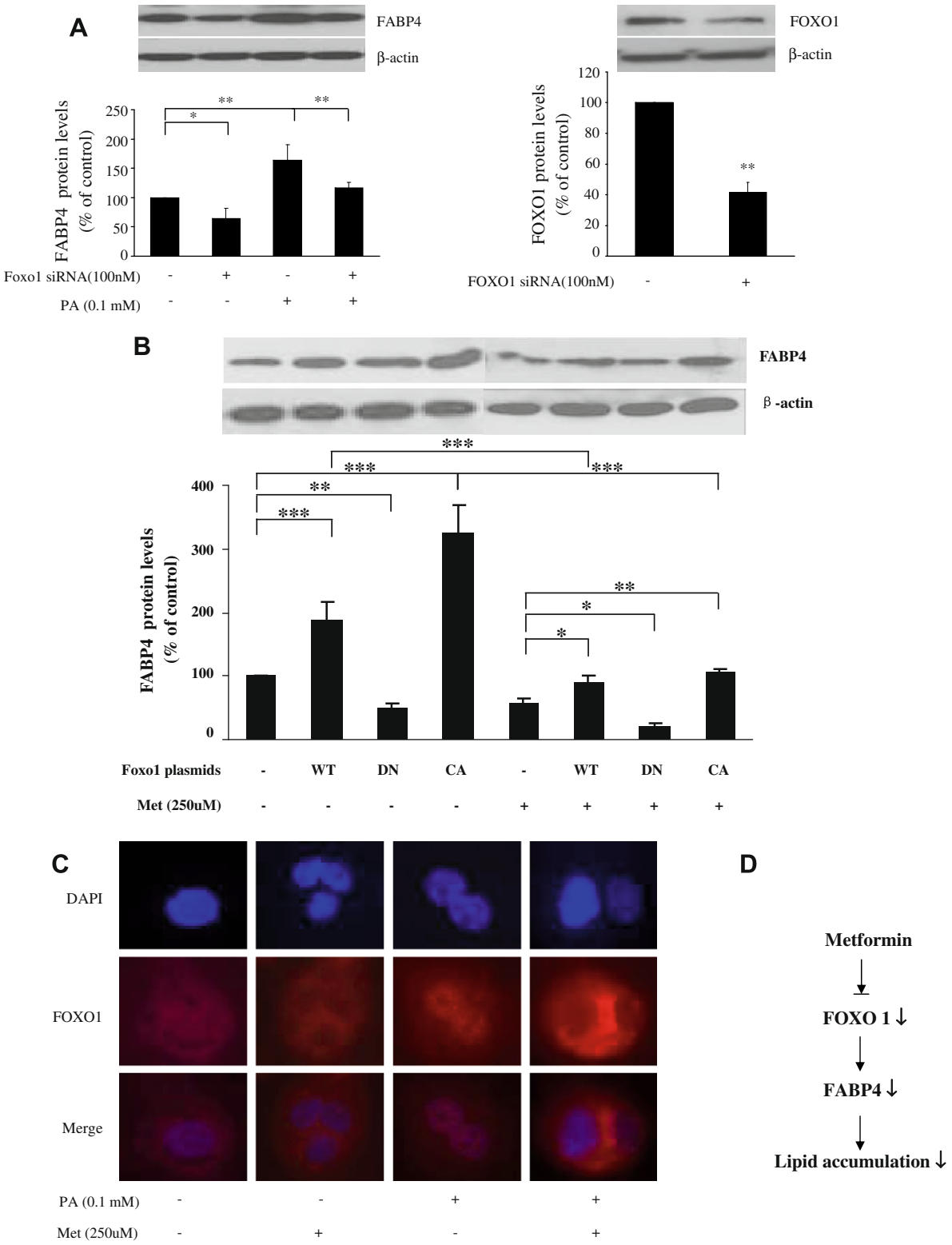

Fig. 4. Metformin reduced FABP4 expression by inhibiting FOXO1. (A) Involvement of FOXO1 in PA-induced FABP4 protein expression. Human THP-1 macrophages were transfected with FOXO1 siRNA and then treated with PA for 24 h. The effectiveness of FOXO1 knockdown was examined by anti-FOXO1 antibody. FABP4 protein was measured by Western blot. The relative levels of protein were compared and expressed as percentages of the control. Representative blots and quantitative analyses from 3 independent experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$ vs the no-treatment control or as indicated. (B) Metformin reduced FABP4 expression by inhibiting FOXO1. THP-1 macrophages were transfected with FOXO1 WT, FOXO1 CA, or FOXO1 DN, followed by treatment with metformin. The expression of FABP4 and β-actin were examined. The relative levels of protein were compared and expressed as percentages of the control. Representative blots and quantitative analyses from 3 independent experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs the no-treatment control, or as indicated. (C) Effects of metformin on FOXO1 cellular location. Human THP-1 macrophage were cultured on coverslips and treated with PA and/or metformin for 24 h. Treated cells were stained with anti-FOXO1 antibody-Texas Red (red) and DAPI (blue). Merged image shows colocalization. Representative images from 3 independent experiments are shown. Metformin promoted FOXO1 nuclear exclusion and prevented PA-induced FOXO1 nuclear translocation. (D) Schematic diagram of the possible mechanism for the metformin-induced reduction in intracellular lipid accumulation in macrophages. Metformin prevented FOXO1 nuclear translocation and subsequently inhibited FOXO1-mediated FABP4 transcription/expression, which leads to decreased intracellular lipid accumulation. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

sion of atherosclerosis. Additionally, studies are necessary to investigate the underling mechanisms in FOXO1-mediated regulation of FABP4 expression and define the detailed signaling involved in metformin-induced inhibition of FOXO1.

Conclusions

In summary, we observed that metformin reduces PA-induced lipid accumulation in macrophages by inhibiting FOXO1-mediated FABP4 expression. Thus, metformin may have therapeutic potential in preventing the accumulation of lipids in macrophages and the formation of atherosclerotic lesions.

Disclosures

None.

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