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Metformin ameliorates Ox-LDL-induced foam cell formation in raw264.7 cells by promoting ABCG-1 mediated cholesterol efflux

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ABSTRACT

Aims: The accumulation of lipids in macrophages contributes to the development of atherosclerosis. Cholesterol efflux of lipid-loaded macrophages mediated by ATP binding cassette (ABC) cholesterol transporters, on the other hand, has been shown to attenuate atherosclerosis progression in patients with unknown mechanism. We therefore sought to test the effect of metformin that reduced cardiovascular risk in diabetic patients independent of its hypoglycemia effect on cholesterol transport in murine raw264.7 macrophages.

Materials and methods: Mouse raw264.7 macrophages were loaded with Ox-LDL (50 μg/ml) for 24 h before incubated with metformin (15 μM) for 24 h. Foam cell formation was assessed by Oil red staining and BIODIPY fluorescent staining as well as cholesterol-ester quantification by commercial kit. Cholesterol uptake and expression of scavenger receptors were detected by flow-cytometry. Cholesterol efflux capacity was measured by fluorescent plate-reader and ABC transporters were detected by Western Blots. Cytokines were detected by ELISA in supernatants and normalized by cellular lysates.

Key findings: Our results showed that metformin decreased oxidized low-density lipoprotein (Ox-LDL)-induced cholesterol accumulation and foam cell formation by increasing cholesterol efflux to HDL, which was associated with an upregulation of ABC transporter ABCG-1. Moreover, metformin increased Ox-LDL-impaired IL-10 secretion, an important anti-foam cell cytokine in atherosclerosis.

Significance: Our data highlighted the therapeutic potential of targeting macrophage cholesterol efflux with new or existing drugs for the possible reduction of foam cell formation in the prevention and treatment of diabetes-accelerated atherosclerosis.

1. Introduction

Atherosclerotic cardiovascular condition accounts for a high proportion of disability and death in diabetic patients [1,2]. Several studies indicated that in poorly controlled diabetes mellitus, altered insulin signaling and/or hyperglycemia promoted unbalanced cholesterol metabolism, which favors Ox-LDL-induced cholesterol retention in cells and macrophage-derived foam cell formation, a hallmark of the initiation and development of atherosclerosis [3–5].

Increased scavenger receptor expression and decreased ABC transporter expression promote macrophage foam cell formation and are considered as a link between diabetes mellitus and atherosclerosis. Studies have found that scavenger receptors SR-A and CD36, which are critical for the uptake of modified lipoproteins [6,7], were upregulated by high glucose concentrations and/or insulin resistance in smooth muscle cells [8]. In addition, the expression of ABC transporters including ABCA-1 and ABCG-1, which mediate intracellular cholesterol removal, were downregulated in response to high glucose [9–11]. Because the accumulation of lipid-loaded macrophages (foam cells) in the atherosclerotic plaques directly contributes to the progression of the disease, the strategies aimed at lowering the lipid content of foam cells may be potential therapeutically.

Metformin has been used extensively as the first-line medication for treating type 2 diabetes mellitus (DM2) during the past 50 years [12,13]. Beyond its anti-hyperglycemic effects, the drug has the advantage of counteracting diabetes-associated cardiovascular complications as reported in large cohorts of individuals, indicating metformin as a suppressor of atherosclerosis through unknown mechanisms [14,15]. Studies have found that metformin reduced palmitic acid -induced foam cell formation in human monocytic cell line THP-1 cells

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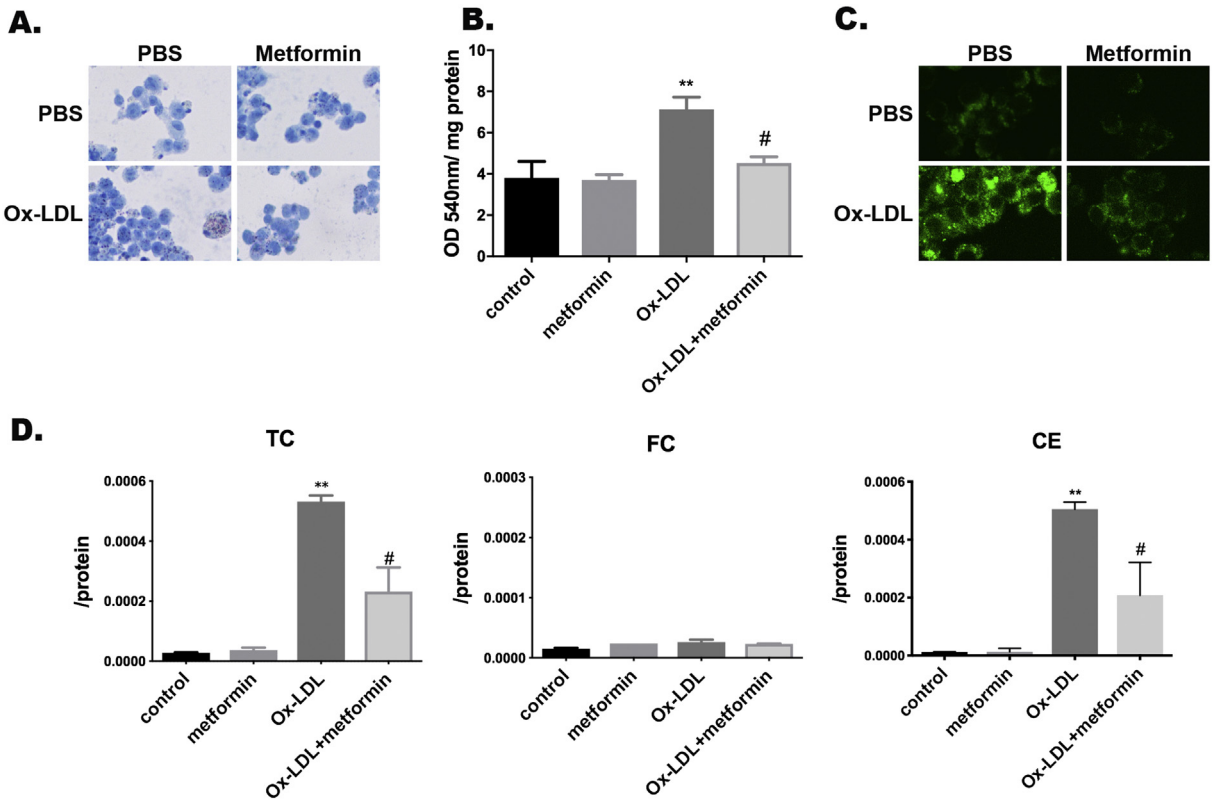

Fig. 1. Metformin attenuates foam cell formation in vitro. A) Representative Oil red O photographs of raw264.7 mouse cells upon 48 h lipid loading with Ox-LDL treated with or without 15 μM metformin for 24 h (200× magnification). B) Quantification of lipid content after alcohol extraction upon Oil red O staining. C) BIODIPY 493/503 staining of macrophage foam cells in the presence or absence of metformin treatment. D) The intracellular total cholesterol (TC) and free cholesterol (FC) content were measured under the same conditions. Cholesterol-ester (CE) was obtained by subtracting FC from TC. Cholesterol content was normalized to cellular protein.
**P < 0.01 vs. control, # P < 0.05 vs. Ox-LDL.

[16]. However, the role of metformin in regulating cholesterol metabolism in Ox-LDL-induced foam cells has not been studied. Since Ox-LDL plays a key role in the progression of atherosclerosis and diabetes complications [17], a test of metformin's direct effect on Ox-LDL-induced foam cell formation is desirable. Our investigation showed that metformin attenuated Ox-LDL-induced foam cell formation in raw264.7 macrophages by promoting ABCG-1-specific cholesterol efflux to HDL, which was associated with a significant increase in ABCG-1 expression.

2. Materials and methods

2.1. Cell culture

Raw264.7 cells, a murine macrophage cell line was obtained from the KeyGEN BioTECH (Jiangsu, China) and grown in high glucose DMEM medium (Gibco, USA) supplemented with 10% (vol/vol) fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, USA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air and grown to 70%–80% confluence.

2.2. Oil red O staining

Cells were loaded with 50 μg/ml human Ox-LDL (Yiyuan Biotechnologies, Guangzhou, China) for 48 h in the presence or absence of 15 μM metformin (Sigma Aldrich, USA) for 24 h. To assess foam cell formation, macrophage slides were fixed with 4% paraformaldehyde and stained with 0.5% Oil red O and hematoxylin. The density of lipid content was evaluated by alcohol extraction after Oil Red O staining. The absorbance at 540 nm was measured with a microplate reader.

2.3. Fluorescent staining

Cells were treated as above. Macrophage slides were fixed with 4% paraformaldehyde and then stained by 2.5 μM BIODIPY493/503(Thermo Fisher, USA). Images were acquired with confocal microscopy scanning.

2.4. Cholesterol content measurement

Cellular total cholesterol and free cholesterol were extracted with lysis buffer provided by the commercial assay kit (Applygen Technologies, China) according to the manufacturer's protocol. The protein concentration of treated cells was determined by BCA assay (Thermo Fisher, USA). The concentrations of total cholesterol and free cholesterol were measured using the absorbance of 530 nm and normalized by protein concentration. Moreover, cholesterol-ester content was obtained by subtracting free cholesterol from total cholesterol for each sample [5].

2.5. NBD-cholesterol uptake

Ox-LDL uptake was measured by determining the uptake of fluorescence-labeled LDL (NBD-LDL; Cayman). Briefly, cells were incubated with Ox-LDL (50 μg/ml) and NBD-cholesterol (5 μg/ml) for 24 h after treatment with metformin or PBS for 24 h. Positive control was established by adding U-18666A (1:1000, Cayman) into the media. Then cells were washed in PBS, digested via trypsin (Gibco, USA) and collected in FACs tubes. After centrifuged at 400*g* in room temperature (RT), supernatants were removed. Cells were then washed by washing buffer (Cayman), centrifuged at 400*g* in RT and resuspended in a final

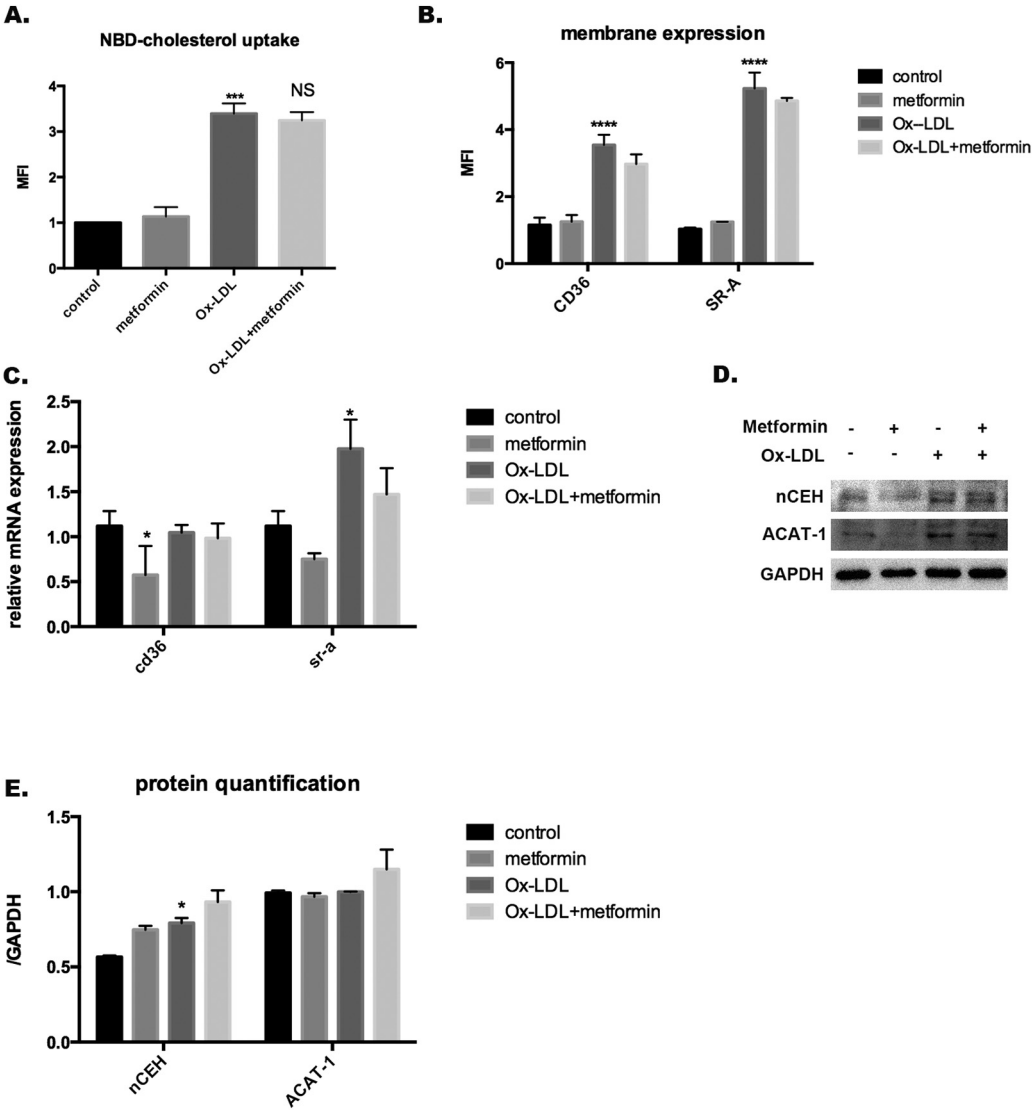

Fig. 2. Metformin did not influence cellular cholesterol uptake or hydrolysis/esterification. A. NBD-cholesterol uptake measured by flow cytometry as mean fluorescent intensity (MFI) in macrophage foam cells in the presence or absence of metformin. B. Expression of surface scavenging receptors mediating cholesterol uptake between treatments. C. Effects of metformin on transcript of scavenger receptors in macrophage foam cells. D & E. Expression and quantification of enzymes involved in cholesterol hydrolysis/esterification between treatments.
*P < 0.05, ***P < 0.001, ****P < 0.0001 vs. control.

volume of 100 μL washing buffer. Cells were analyzed using a flow cytometry (BD FACS Calibur) under FITC fluorescent and fluorescence intensity was calculated as fold increase over unstained genotype and treatment controls by FlowJo software.

2.6. Flow cytometry

Cells were washed in PBS, digested via trypsin, collected in FACs tubes and pelleted at 1000*g* under 4 °C. Cells were then washed in MACS Buffer (Miltenyi Biotec) and pelleted at 1000*g*. Cells were then incubated with antibodies APC-CD36 (1:10; Miltenyi Biotec), FITC-SR-A (1:10; Miltenyi Biotec) in a volume of 50 μL covered from light for 10 min. Cells were then washed twice more with MACS Buffer and re-suspended in a final volume of 500 μL. Cells were analyzed using a flow cytometry (BD FACS Calibur) and fluorescence intensity was calculated as fold increase over unstained genotype and treatment controls by FlowJo software.

2.7. Cholesterol efflux assay

Cells were loaded with 50 μg/ml Ox-LDL and 1 mg/ml NBD-cholesterol (Cayman, USA) in serum-free medium containing 0.2% fatty acid-free BSA (Quintech Inc., USA) for 24 h to equilibrate cellular cholesterol pools. Then, cell layers were rinsed and incubated in the absence or presence of metformin for an additional 6 h. Cholesterol efflux proceeded for 6 h at 37 °C in medium containing 0.2% BSA, 0.2% BSA plus 15 mg/ml lipid-free human ApoA-I (Sigma Aldrich), or 0.2% BSA plus 50 mg/ml of human HDL (Biovision, China). At the end of this incubation, the media was collected and centrifuged at 13,000 rpm for 10 min to remove debris. Cells were lysed with 0.5 ml of 0.1% Triton X-100 and supernatants were collected after centrifuge at 13,000 rpm for 10 min. The fluorescence-labeled cholesterol released from the cells into the medium was measured with a multifunctional microplate reader (Molecular Devices M3). Cholesterol efflux was expressed as the percentage of fluorescence in the medium relative to the total amount of fluorescence (cells and medium). The specific efflux of ApoA-I or HDL was calculated by subtracting non-specific efflux in the presence of

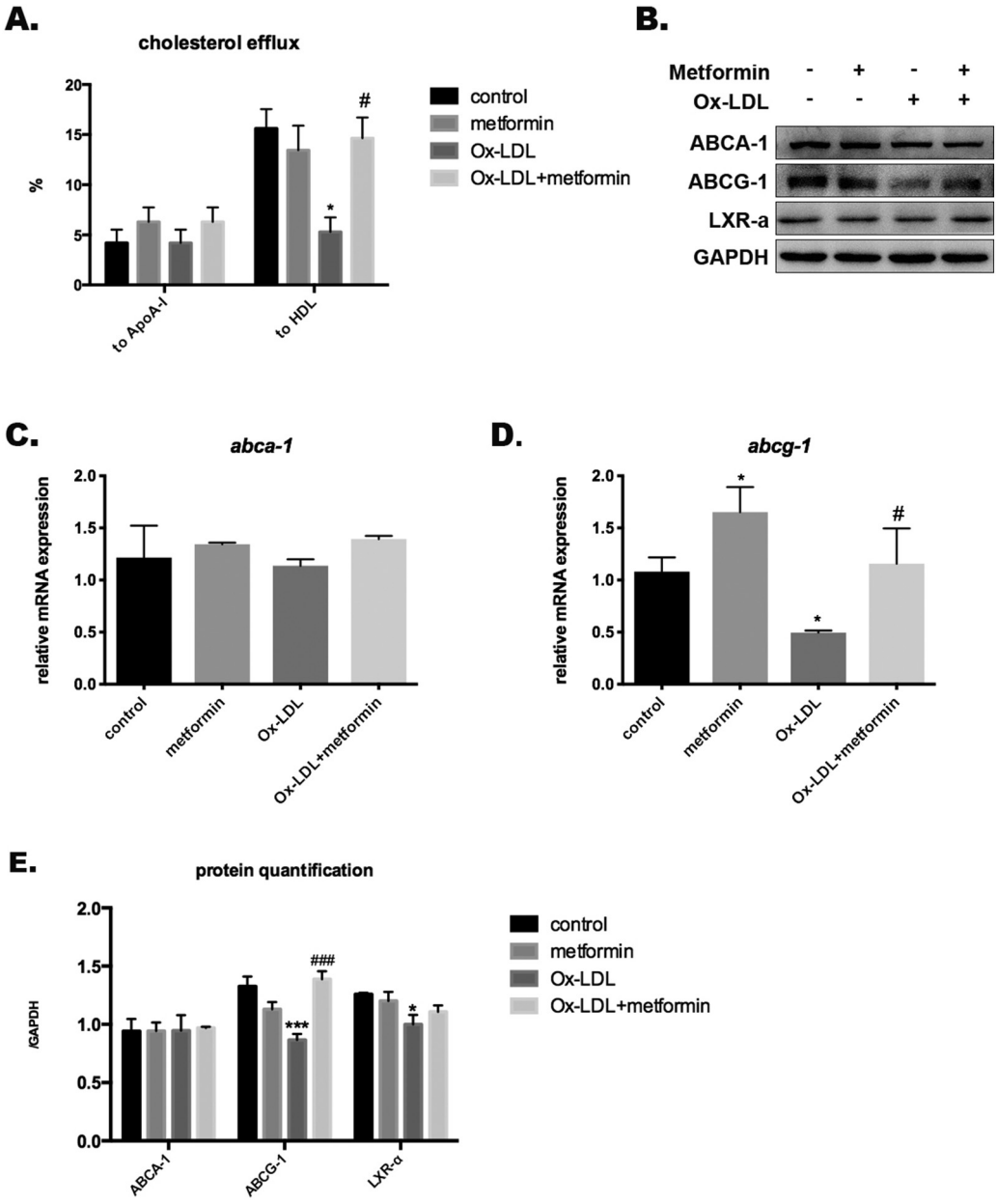

Fig. 3. Metformin increased ABCG-1-mediated cholesterol efflux in macrophage foam cells. A. Effect of metformin on cholesterol efflux to ApoA-1 and HDL in macrophage foam cells. NS: not significant. B & E. Impact of metformin on the expression of cholesterol efflux transporters in macrophage foam cells. C. Transcriptional levels of cholesterol efflux transporters in macrophage foam cells treated with or without metformin.
*P < 0.05, ***P < 0.001, ****P < 0.0001 vs. control; #P < 0.05, ####P < 0.0001 vs. Ox-LDL.

0.2% BSA only [18].

2.8. Western Blot

Macrophages were washed twice with PBS and harvested in lysis buffer (RIPA with Protease and Phosphatase Inhibitors) on ice for immunoblotting. Mixture were collected and centrifuged at 13,000*g* for 15 min at 4 °C, and the resulting supernatants were used as the cellular lysates. Aliquots (30 μg) of cell lysates were separated on 10% SDS-PAGE and then transblotted onto the PVDF membrane (Millipore). After being blocked with 5% BSA, blots were incubated with various primary rabbit antibodies including anti-ABCG-1 (Protein-tech), anti-ABCA-1 (SAB), anti-LXR-α (Protein-tech), anti-nCEH (Abcam), anti-ACAT-1 (Abcam), anti-P-AMPKα (phospho Thr172) (#2535,CST), anti-GAPDH (Bio-world) and followed by horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody(Bio-world). secondary antibodies. The protein bands were detected by ECL using a Syngene system. Protein quantification was measured by using Image J software and normalized by GAPDH.

2.9. Total RNA isolation and real-time PCR

Total RNA was extracted from macrophages using an RNeasy kit (Qiagen) guided by instructions of the manufacturer. cDNA was prepared using a cDNA reverse transcription kit (Takara) according to the instructions of the manufacturer. mRNA expression was measured on the RT-PCR system (Applied Biosystems) using TaqMan primers for mouse CD36, SR-A, ABCA-1, ABCG-1 and GAPDH. Fold changes in expression were calculated by the Ct method using mouse GAPDH as an endogenous control for mRNA expression. All-fold changes are expressed normalized to the untreated control.

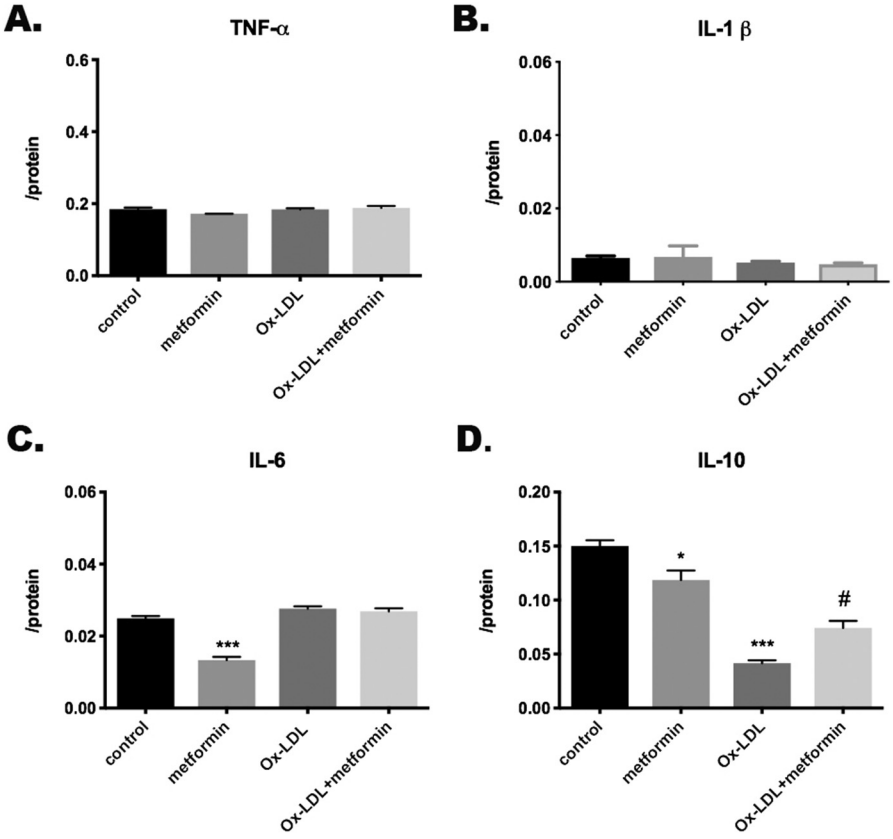

Fig. 4. Metformin improved Ox-LDL-impaired anti-foam cytokine secretion in macrophage foam cells. A–D) Raw264.7 macrophages were cultured with or without metformin and the pro- and anti-foam cytokines were detected.
*P < 0.05, ***P < 0.001 vs. control; #P < 0.05 vs. Ox-LDL.

2.10. Primer sequence

Gene	Accession number	Primer sequence
ABCA-1	NM_013454.3	Forward: gcagatcaagcatcccaact Reverse: ccagagaatgtttcattgtcca
ABCG-1	NM_009593.2	Forward: gggtctgaactgccctacct Reverse: tactcccctgatgccacttc
CD36	NM_007643.4	Forward: ttgtacctatactgtggc Taaatgaga Reverse: tctaccatgccaaggagctt
SR-A	NM_031195.2	Forward: gcatcccttcctcacagc Reverse: aatgagggcagccttgaa
GAPDH	NM_008084.2	Forward: agcttgtcatcaacgggaag Reverse: tttgatgttagtggggtctcg

2.11. Cytokines measurement (ELISA)

Cultured media were collected and centrifuged at 8000*g* for 5 min at room temperature to remove cellular debris. Supernatants were used to determine cytokine levels of TNF-α, IL-1β, IL-6 and IL-10 according to the instructions of the manufacturer and were normalized by protein concentration of cellular lysates.

2.12. Statistical analysis

All results are shown as mean ± SD. Results were analyzed using 1-way ANOVA by GraphPad Prism software. A Tukey post hoc test was used to test for significant differences revealed by the ANOVA. Significance was accepted at $P \leq 0.05$.

3. Results

3.1. Metformin attenuated Ox-LDL-induced foam cell formation in raw264.7 macrophages

To test the effect of metformin on macrophage foam cell formation, we initially treated Raw264.7 macrophages with Ox-LDL for 24 h, and then tested cells in the presence or absence of metformin for additional 12 h, 24 h and 48 h. Oil red O and fluorescent staining both showed that the macrophages exposed to Ox-LDL exhibited a foam cell phenotype characterized by lipid droplet formation, which was ameliorated by metformin treatment for 24 h (Fig. 1A and C). 12 h metformin only showed minimum effect, whereas 48 h metformin resulted in a significant cell death in the culture (data not shown). Thus, 24 h metformin treatment was adopted for the study. Again, analysis of cellular lipid density by alcohol extraction indicated that 24 h metformin decreased lipid deposition in Ox-LDL-induced macrophage foam cells (Fig. 1B). Cholesterol quantification tests further showed that the 24 h metformin-treated macrophage foam cells had almost 50% less total cholesterol content and 55% less cholesterol-ester levels (Fig. 1D) than those of control macrophages maintained in metformin-free media. Together, these data indicated a direct effect of metformin in reducing intracellular lipid accumulation on macrophages in vitro.

3.2. Metformin has little inhibitory effect on cholesterol uptake and hydrolysis/esterification

Since scavenger receptors SR-A and CD36 have been demonstrated to be the main effectors by which Ox-LDL is taken into macrophages [7], we therefore investigated the role of metformin in regulating scavenger receptors-mediated Ox-LDL uptake. Flow cytometry results

showed no detectable changes in surface expressions of SR-A and CD36 in metformin-treated foam cells (Fig. 2B), so was in transcripts of the two receptors shown in Fig. 2C. Functional analysis further supported the notion that metformin has little impact on Ox-LDL uptake in macrophages (Fig. 2A). We then tested the regulatory role of metformin in intracellular lipid droplet formation, during which free cholesterol, the hydrolyzed product of Ox-LDL mediated by nCEH in cells during lipid uptake, was re-esterificated to cholesterol-ester by ACAT-1 for lipid storage [19]. Western Blots showed that the expression of nCEH and ACAT-1 were not affected by metformin treatment in macrophage foam cells (Fig. 2D & E). Thus, metformin had little effect on hydrolysis and esterification of cholesterol. Taken together, these results suggested that the rate of cholesterol uptake as well as intracellular lipid conversion and storage in the testing cells was hardly influenced by metformin.

3.3. Metformin improved Ox-LDL-impaired cholesterol efflux by increasing ABCG-1 expression

We then tested whether metformin decreased intracellular lipid deposition by improving cholesterol efflux to extracellular acceptor HDL and/or lipid-poor ApoA-I, a process mediated by ABCG-1 and/or ABCA-1, respectively [10,20,21]. Functional analysis showed that metformin treatment indeed resulted in an increase of cholesterol efflux to HDL, but not to ApoA-I in lipid-loaded macrophages (Fig. 3A). In consistent with this observation, we found a strong reverse of Ox-LDL impaired ABCG-1 expression both at transcript and protein levels, while the levels of ABCA-1 transcripts and proteins were unaffected (Fig. 3B, C & E). Moreover, there was no difference in the expression of nuclear receptor LXR-α, which was reported to upregulate ABCA-1-mediated cholesterol efflux [17]. We further examined AMPK pathway, which mediates the action of suppressing hepatic glucose production by metformin [22], in the regulation of ABCG-1. Our results showed that while metformin induced the phosphorylation of AMPK in macrophages, it had little effect on AMPK phosphorylation in foam cells, suggesting that metformin may regulate ABCG-1 via AMPK independent mechanisms. Together, these results implied that metformin may specifically improve ABCG-1-mediated cholesterol efflux, supporting the notion that cholesterol efflux capacity represents a strong independent risk marker inversely associated with cardiovascular disease [23].

3.4. Metformin ameliorated Ox-LDL induced pro-foam cell status of raw 264.7 cells

A large number of cytokines are expressed in atherosclerotic lesions [24–27] and facilitate a diverse range of functions that impact on disease progression and macrophage foam cell formation. To examine how metformin modulated the secretion of pro or anti-foam cell cytokines in Ox-LDL-loaded macrophages, the conditional media were collected for cytokine measurement. ELISA results showed that TNF-α, IL-1β and IL-6, which were shown to promote foam cell formation [28–30], were slightly increased in macrophage foam cells in vitro and seemed unchanged after metformin treatment (Fig. 4A–C). However, Ox-LDL significantly downregulated IL-10 secretion, an anti-inflammatory cytokine reported to reduce lipid accumulation and atherosclerosis in vivo [31,32], and metformin largely reversed its reduced levels (Fig. 4D). These data indicated that IL-10, other than pro-inflammatory cytokines, may play a more important role in metformin regulation of foam cell formation.

4. Discussion

T2DM significantly increases the risk for the development of atherosclerosis, primarily due to the imbalance of cholesterol influx [6] and efflux [9] in macrophages, leading to increased intracellular cholesterol-ester accumulation. Cardiovascular benefits of metformin in diabetic patients were observed in clinical trials but its effects on cholesterol metabolism of macrophage foam cells remained elusive. Our data found that metformin reduced Ox-LDL-induced cholesterol-ester deposition in macrophages by specifically upregulating cholesterol efflux without influencing influx in vitro.

CD36 and SR-A play a main role in extracellular modified LDL uptake by macrophages via receptors-mediated phagocytosis and pinocytosis [7,33]. In particular, CD36 is responsible for > 50% of the oxidized-LDL uptake [34] and high glucose induces its expression post-transcriptionally [35]. Similarly, we found that CD36 expression was upregulated by Ox-LDL at post-transcriptional level. Previous study has shown that macrophages from mice lacking SR-A and/or CD36 showed reduced Ox-LDL internalization and were less prone to foam cell formation in vitro [24]. However, metformin treatment did not significantly downregulate the expression of SR-A and CD36 as well as Ox-LDL uptake into macrophages, implying that metformin is likely regulating macrophage foam cell formation via uptake-independent mechanisms.

Cholesterol efflux, the process of cholesterol-esters export from cholesterol-loaded macrophages, was strongly reversely associated with cardiovascular risks [36–39]. Members of the ABC transporter superfamily are known regulators of cholesterol efflux as ABCA-1 mediates cholesterol efflux to lipid-free ApoA-I and ABCG-1 to HDL. Our observation that expression of ABCG-1, but not ABCA-1, was reduced in response to Ox-LDL supported the finding that cholesterol efflux mediated by HDL, but not ApoA-I, was inhibited. Consistently, targeted disruption of ABCG-1 in mice was reported to cause massive lipid accumulation in multiple tissues including macrophages, which was prevented by over-expression of ABCG-1 [10]. In addition, previous studies showed that macrophages from T2DM patients [9] and diabetic mice [40] both have an increased activation of proatherogenic pathways because of the significant reductions in ABCG1-specific cholesterol efflux. This suggested that ABCG-1 played an important role in regulating lipid homeostasis and Ox-LDL-induced downregulation of ABCG-1 was involved in macrophage lipid accumulation. Our results showed that upregulation of ABCG-1 expression by metformin was accompanied by increased cholesterol efflux to HDL and decreased lipid accumulation in the macrophages, implying a suppressive effect of metformin on macrophage foam cell formation. We then examined how metformin regulated ABCG-1 by testing AMPK pathway, which played an important role in hypoglycemia action of metformin [22]. We found that the phosphorylation of the AMPK α-subunit at T172, which leads to an increase in AMPK enzymatic activity, was not induced by metformin in foam cells, suggesting more complicated mechanisms other than AMPK pathway were involved in the action of metformin. More recently, autophagy has drawn great interest in cardiovascular research and a small amount of studies showed that the promotion of macrophage autophagy increased cholesterol efflux [41,42], thus raised the possibility that metformin may regulate ABCG-1 expression by autophagy-related mechanisms.

A number of cytokines are emerging as regulators of atherosclerosis. For example, IL-1β and IL-6 were found to promote atherosclerosis in mice [43–45] while IL-10 reduced atherosclerosis in vivo [31,32]. Moreover, these cytokines were reported to directly modulate macrophage foam cell formation. Studies have shown that TNF-α and IL-1β both promoted foam cell formation in vitro by reducing ABCA-1/ABCG-1 mRNA expression [30,46]. IL-6 was reported to enhance Ox-LDL uptake and CD36 mRNA levels in mouse peritoneal macrophages in vitro while Liao et al. [47] demonstrated that it decreased foam cell formation by reducing SR-A mRNA levels and SR-A promoter activity in human macrophages [48]. IL-10 was found to down-regulate CD36 mRNA expression and enhance ABCA-1/ABCG-1 expression levels in human macrophages [49]. In our experiments, metformin improved Ox-LDL-impaired IL-10 secretion while TNF-α, IL-1β and IL-6 levels seemed unchanged. Whether this effect of anti-foam cell formation by

metformin relies on IL-10 upregulation requires further investigation.

5. Conclusion

Metformin promoted Ox-LDL-impaired ABCG-1 expression, sequentially upregulated ABCG-1-specific cholesterol efflux to HDL, leading to reduced foam cell formation in raw264.7 macrophages. Upregulation of IL-10 secretion was also observed.

Conflicts of interests

The authors declare that there are no conflicts of interest.

Acknowledgments

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