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Intermedin Ameliorates Atherosclerosis by Increasing Cholesterol Efflux Through the cAMP-PKA Pathway in Macrophage RAW264.7 Cell Line

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Data Interpretation D
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Background: The aim of this study was to explore the role of intermedin and its mechanism in cholesterol efflux of macrophage THP-1 and RAW264.7 cell lines in atherosclerosis (AS).




Material/Methods: ApoE^{-/-} mice were fed with a high-fat diet, and the concentrations of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured. The lipidoses of the aortic sinus were analyzed by hematoxylin and eosin staining, and the cAMP level was detected by enzyme-linked immunosorbent assay (ELISA). The expressions of ATP-binding cassette transporter (ABCA1) were tested by real-time PCR and Western blot analysis.

Results: IMD decreased serum TC and LDL-C, and increased serum HDL-C level in apoE^{-/-} mice and attenuated AS plaque areas. *In vitro*, IMD increased intracellular cAMP concentration in a dose-dependent manner in THP-1 and RAW264.7 cell lines, which enhanced the expression of ABCA1 and increased cholesterol efflux rate. However, this effect was inhibited by PKAI in the RAW 264.7 cell line but not in the THP-1 cell line.

Conclusions: IMD can ameliorate the development of AS in ApoE^{-/-} mice and regulate cholesterol balance in the RAW264.7 cell line through the cAMP-PKA pathway.

MeSH Keywords: **Atherosclerosis • ATP Binding Cassette Transporter 1 • Cyclic AMP-Dependent Protein Kinase R1alpha Subunit • Cyclic AMP-Dependent Protein Kinases**

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Background

Atherosclerosis (AS) is one of the leading causes of death among cardiovascular diseases, which is attributed to the uptake of oxidized low-density lipoprotein (ox-LDL) by macrophage and foam cell formation that are accumulated by intracellular cholesterol in the artery wall [1]. Macrophage-derived foam cell formation is considered to be a hallmark in this process [2]. High-density lipoprotein (HDL) is believed to play a protective role in transporting cholesterol to the liver from the peripheral tissues, and an elevated level of HDL is associated with a lower level of cholesterol in foam cells [3]. ATP-binding cassette transporter A1 (ABCA1) is a first and rate-limiting step of this reverse cholesterol transport (RCT) process, which is also a key mediator in regulating cellular cholesterol homeostasis expressed on the fat-laden macrophage surface [4,5]. Mutations of ABCA1 genes are known to have a severe deficiency of HDL, thus resulting in AS [6]. As the cAMP-inducible apolipoprotein receptor, ABCA1 promotes lipids secretion from the macrophages, and is also phosphorylated by protein kinase A (PKA) or a PKA-like kinase *in vivo* [7,8]. Hu et al. reported that PKA is involved in the ABCA1-mediated cholesterol efflux [9]. Nevertheless, whether the cAMP-PKA-ABCA1 pathway is feasible in the setting of increasing cholesterol efflux is not fully understood.

Intermedin (IMD), an intrinsic active polypeptide (also known as adrenomedullin-2), is a newly identified member of the calcitonin gene-related peptide (CGRP) superfamily that was found by Roh et al. [10]. IMD is widely distributed in the peripheral and central nervous system of humans and mice and is known for its antioxidant and antiinflammatory properties. Other biological effects associated with IMD include facilitating vessel dilation, inhibiting vessel calcification, and enhancing myocardial contraction [11]. Recent studies have shown that exogenous IMD treatment plays a protective role in AS by ameliorating AS plaque in ApoE^{-/-} mice by inhibiting SR-A and CD36 in macrophages and decreasing foam cell formation, indicating that IMD functions in regulating lipid metabolism and delaying AS development [12,13].

However, the potential molecular mechanism of the anti-AS function of IMD remains unclear. In the present study, we investigated the underlying mechanism of the effect on ABCA1 expression and ABCA1-mediated cholesterol efflux by IMD in the RAW264.7 cell line, which are mouse macrophage-derived foam cells. We demonstrate that IMD significantly increased ABCA1 expression through the cAMP-PKA signaling and decreased cholesterol retention in the RAW264.7 cell line. Here, we provide a novel mechanism of the cAMP/PKA-mediated cholesterol efflux by IMD administration and explain its role in anti-AS function.

Material and Methods

Animals

Twenty-four ApoE^{-/-} mice (male, 8 weeks old, 18–20 g) were purchased from the Institute of Model Animals in Nanjing University and housed in a pathogen-free animal facility at West China Hospital of Sichuan University. Mice were fed with a high-fat diet (15.8% fat and 1% cholesterol) for 10 weeks to produce AS and then were equally divided into 3 groups: normal saline (N=8), 100 ng/kg/h of IMD (N=8), and 500 ng/kg/h of IMD (N=8). All mice were implanted with IMD for 6 weeks by an osmotic pump under local anesthesia. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at West China Hospital of Sichuan University.

Cell culture and transduction

The human macrophage cell line THP-1 and the mouse macrophage cell line RAW264.7 were obtained from the American Type Culture Collection. THP-1 was cultured in 1640 medium with 10% fetal bovine serum (FBS) (Gibco), supplemented with 1% penicillin-streptomycin (Hyclone, USA) in a humidified incubator containing 5% CO₂ at 37°C. We seeded and induced 2.5×10⁵ cells using 50 ng/ml of phorbol myristate acetate (PMA). Macrophage THP-1 was successfully induced by observing the cells attached to the bottom. RAW264.7 was cultured in Dulbecco modified Eagle's medium (DMEM, Gibco, USA) in 10% FBS (Gibco) and supplemented with 1% penicillin-streptomycin (Hyclone) in a humidified incubator containing 5% CO₂ at 37°C.

Reagents

IMD was purchased from Phoenix BioTECH (Beijing, China). PMA, 3H-cholesterol, and ApoA-I were purchased from Sigma. The cAMP parameter assay kit was obtained from R&D Systems (Shanghai, China). Trizol reagent was obtained from Invitrogen, USA. The antibodies against ABCA1 (ab18180) and β-actin (ab8227) were purchased from Abcam (Shanghai, China). HE staining reagent was bought from Beyotime Biotechnology (Haimen, China).

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from THP-1 and RAW264.7 cells. We reverse transcribed 1 μg of total RNA using iScript™ reverse transcription (Bio-Rad, USA), as recommended by the manufacturers. Expression of mABCA1 and hABCA1 and the internal control m-β-Actin and h-β-Actin mRNA levels were analyzed using quantitative real-time RT-PCR on an iCycler thermocycler (Bio-Rad). TaqMan assays were obtained from Applied Biosystems

(USA). The relative quantities of mABCA1 and hABCA1 were calculated using iCycler iQ Real-Time Detection System software (version 3.0a; Bio-Rad, USA) through the comparative threshold method ($\Delta\Delta Ct$), using m- β -Actin and h- β -Actin as endogenous controls. The sequences for mABCA1 were: forward: 5'-GGGTCTGAACTGCCCTACTCT-3', reverse: 5'-TACTCCCCTGATGCCACTTC-3'; hABCA1 were: forward: 5'-GCCTGCTAGTGGT CATCTG -3', reverse: 5'-CCACGCTGGGATCACTGTA-3'; m- β -Actin, forward: 5'-CTAAGGCCAACCGTGAAAG-3' and reverse: 5'-ACCAGAGGCATACAGGGACA-3'; h- β -Actin, forward: 5'-CCAAC CGCGAGAAGATGA-3' and reverse: 5'-CCAGAGGCGTACA GGGATAG-3'.

Cholesterol efflux rate analysis

Counts per minute (CPM) was used to measure the medium and cell-associated [3H] cholesterol. The cholesterol efflux rate was calculated by the following equation: $[\text{total media counts}/(\text{total cellular counts} + \text{total media counts})] \times 100\%$. The procedure followed has been detailed by a prior study [14].

Western blot

Cells were lysed in a sodium dodecyl sulfate (SDS) sample buffer by adding one-third the volume of a 4 \times SDS sample buffer directly into the cell suspensions. The samples were then boiled for 5 min, and 20 μ g was loaded onto 12% SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, USA). Immunoblotting was performed using first antibody overnight at 4°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control of cytoplasmic protein. The blots were detected using enhanced chemiluminescence reagent (ECL, Thermo, USA). The amount of each target gene was normalized by the level of GAPDH in each sample. The experiments were repeated at least 3 times.

Enzyme-linked Immunosorbent Assay (ELISA)

The cAMP concentrations in cell culture supernatants were quantified using commercially available Quantikine ELISA kits (R&D Systems, USA) according to the manufacturer's instructions and as described in our previous publications. Briefly, the plates were coated overnight with specific antibodies to cAMP, and the following morning the plates were washed and blocked for 2 h. Samples were added to the wells at various dilutions and then incubated with detection antibody for 2 h. Plates were subsequently washed, and streptavidin-HRP conjugate cAMP was added to each well for 15 min. This was followed by an additional washing step and plates were incubated with a substrate solution. The enzymatic reaction was then quantified by measuring absorbance at 450 nm using a standard plate reader.

Lipid profile measurement

A blood sample was extracted from the eyeball after 12-h fasting. Serum was obtained by centrifugation at 4000 rpm for 15 min at 4°C and preserved at -80°C before analysis. Serum lipoproteins levels were measured enzymatically using an automatic biochemistry analyzer (Hitachi7600, Japan).

HE staining

The tissue sections were deparaffinized in xylene, rehydrated with a graded series of ethanol, and then endogenous peroxidase activity was blocked by a 5-min incubation in 3% hydrogen peroxide in methanol. After two 5-min washes with phosphate-buffered saline, an avidin-biotin complex and immunoperoxidase were applied. Slides were then washed in isopropanol for 10 min, rinsed in tap water, counterstained with hematoxylin and mounted in a glycerol/gelatin solution. Images of cells were then captured using an Olympus light microscope (Tokyo, Japan).

Statistical analysis

Data are expressed as mean \pm SD, and statistical analysis was performed via a *t* test or one-way analysis of variance. A *p* value less than 0.05 was considered statistically significant.

Results

IMD regulates lipid metabolism and attenuates AS in ApoE $^{-/-}$ mice

To determine the effects of IMD on the lipid metabolism in AS *in vivo*, ApoE $^{-/-}$ mice were fed a high-fat diet for 10 weeks to develop AS. The mice were divided into groups based on the IMD dose, then we measured the levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and HDL-C. We observed that TC decreased dramatically after high IMD stimulation (9.47 ± 1.20 vs. 11.12 ± 1.42 , $p < 0.05$) (Table 1). High-dose IMD decreased the LDL-C level significantly more than in the low-dose group, while HDL-C increased both in the low-dose and high-dose stimulation. No difference was observed in the TG group ($p > 0.05$). Next, each of the AS plaques were measured by hematoxylin and eosin staining (Figure 1A–1C). We found that the AS plaque was prominently reduced by 37% in the 500 ng/kg/h group and reduced by 18.5% in the 100 ng/kg/h group (Figure 1D). Therefore, IMD-mediated lipid metabolism regulation may help relieve the process of atherosclerosis in ApoE $^{-/-}$ mice.

Table 1. Effects of IMD on lipid metabolism.

Item	Vehicle (n=8)	IMD 100 ng/kg/h (n=8)	IMD 500 ng/kg/h (n=8)	P value
Weight (g, 8 weeks)	18.83±1.04	18.25±0.81	18.64±0.71	0.983
Weight(g, 25weeks)	29.59±2.88	29.16±2.47	28.29±2.05*	0.063
TG (mmol/L)	1.55±0.13	1.53±0.12	1.50±0.16	0.75
TC (mmol/L)	13.71±1.22	11.12±1.42*	9.47±1.20*#	0.001
LDL-C (mmol/L)	5.13±0.86	4.59±0.43	4.19±0.63*	0.034
HDL-C (mmol/L)	0.80±0.08	0.96±0.11*	1.02±0.16*	0.002

Vehicle – using saline as the control group. IMD 100 ng/kg/h – IMD low concentration group; IMD 500 ng/kg/h – IMD low concentration group. Data are presented as mean ±SD. * p<0.05, as compared with Vehicle; # p<0.05, as compared with IMD low concentration group.

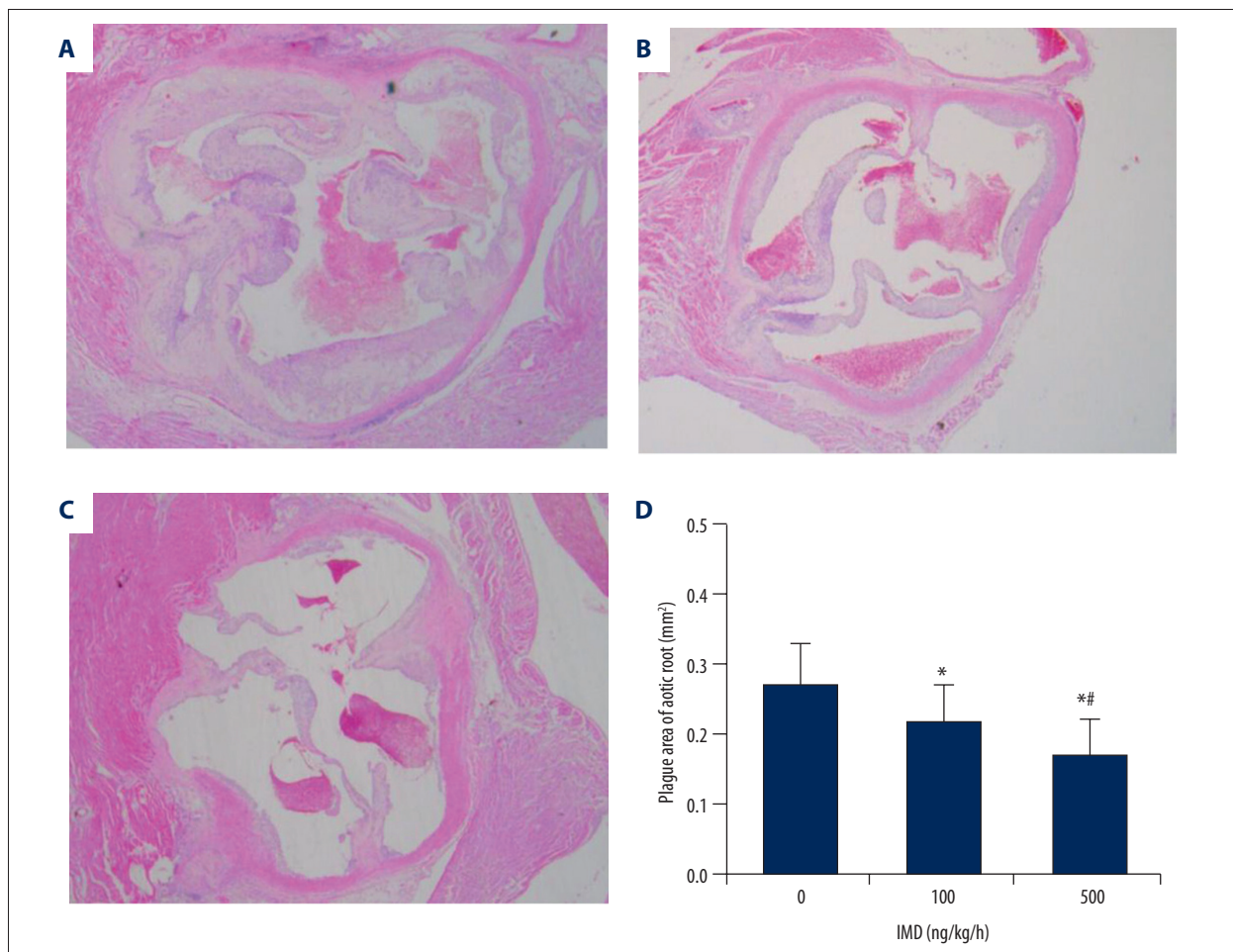


Figure 1. IMD attenuates atherosclerosis. Atherosclerotic plaque was reduced significantly in the 100 ng/kg/h group and the 500 ng/kg/h group. (A) Vehicle control group. (B) IMD 100 ng/kg/h group. (C) IMD 500 ng/kg/h group. (D) Analysis of atherosclerotic plaque between the 3 groups. * Compared with control group (P<0.05) # compared with IMD 100 ng/kg/h group (P<0.05).

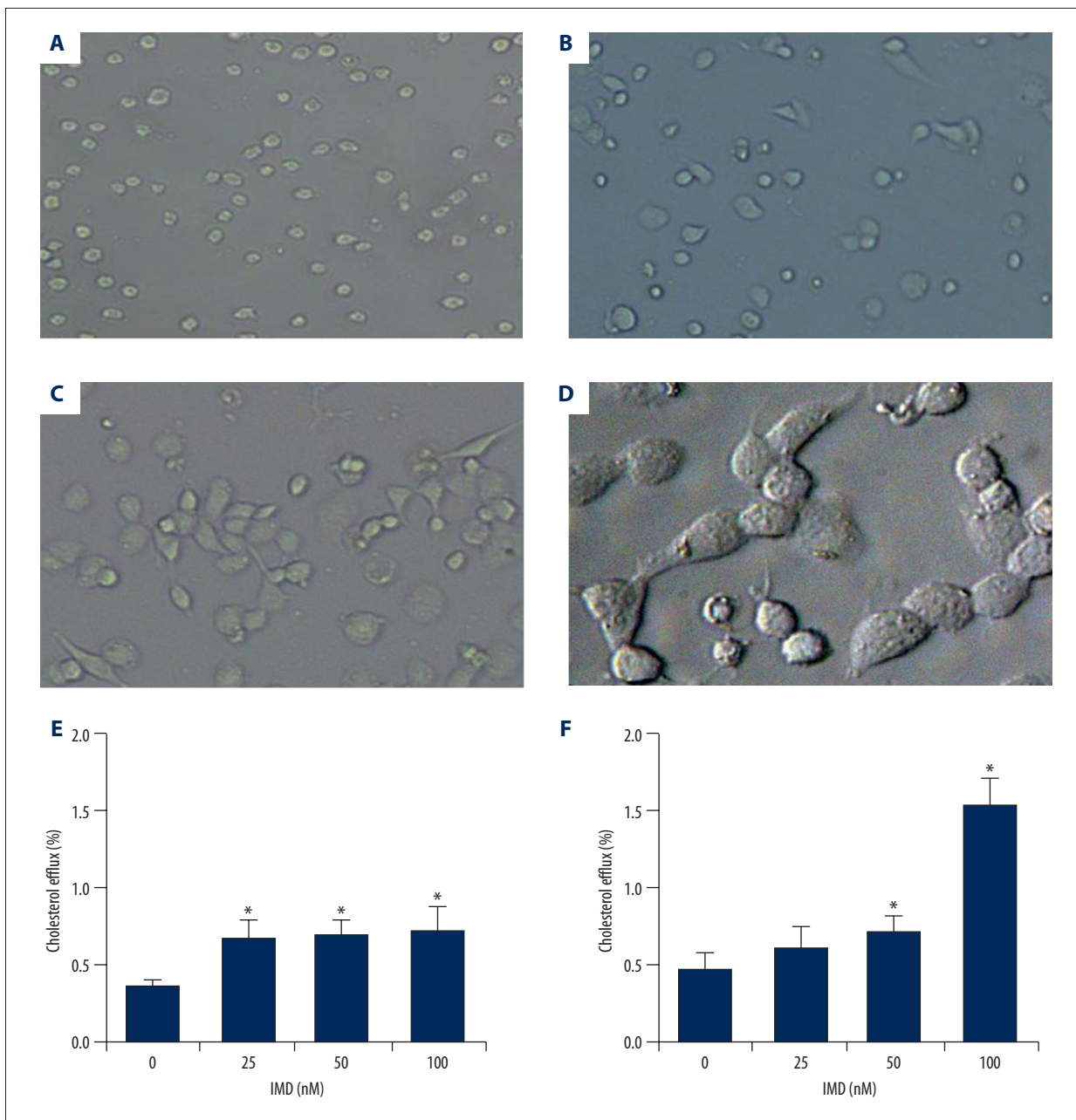


Figure 2. Intermedin facilitates cholesterol efflux in macrophages. Macrophage THP-1 was induced by 50 ng/ml PMA. The morphology of the macrophage THP-1 was observed at 10× and 100× under a microscope and confirmed the transformation in 48 h. Counts per minute (CPM) was used to measure medium- and cell-associated [3H] cholesterol. (A) 0 h 10×. (B) 24 h 10×. (C) 48 h 10×. (D) 48 h 100×. (E) CPM in THP-1. (F) CPM in RAW264.7. (* P<0.05).

IMD facilitates cholesterol efflux in macrophages

IMD enhanced the expression of HDL in ApoE^{-/-} mice, which is related to a lower level of cholesterol in the foam cells. Therefore, we explored how the cholesterol efflux in IMD influences cholesterol balance in macrophages. Macrophage THP-1 was transferred successfully from its floating monocyte type by 50-ng/ml PMA inducement. The morphology of

the macrophage THP-1 was observed under a microscope at 10X and 100X and we confirmed the transformation at 48 h (Figure 2A–2D). THP-1 and RAW264.7 cells were pretreated with 3H-cholesterol medium for 24 h and then treated with IMD at different concentrations for another 24 h. We added 10 µg/ml ApoA1 for another 24 h after IMD stimulation. As shown in Figure 2E, 2F, the CPM of cholesterol was double that of the counterparts in the control group (0.73±0.14 vs. 0.36±0.05,

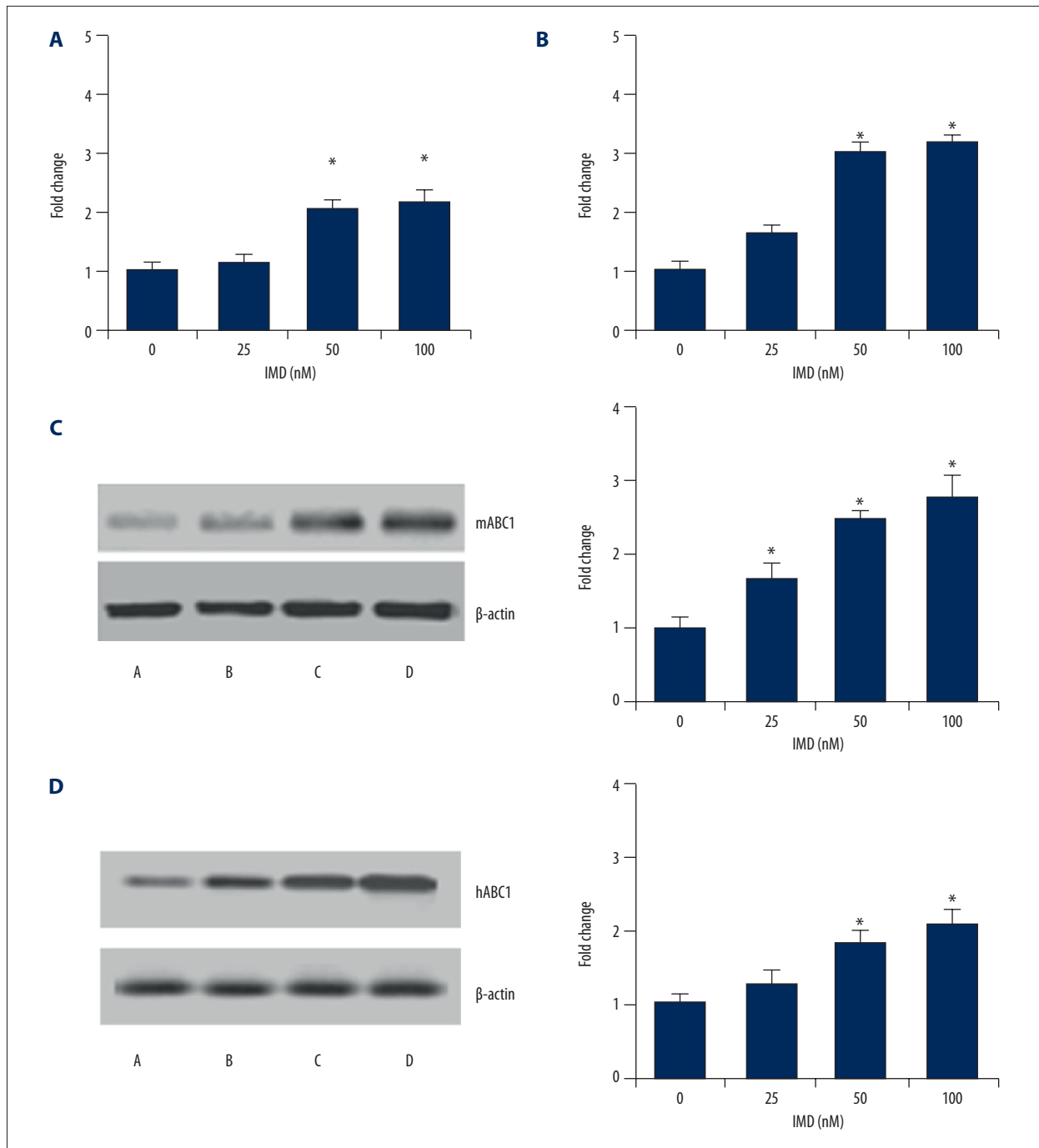


Figure 3. Intermedin promotes the expression of ABCA1. Cell mRNA and protein of ABCA1 was extracted and measured by PCR and Western blot. (A) mRNA of ABCA1 in THP1. (B) mRNA of ABCA1 in RAW264.7. (C) Protein of ABCA1 in THP1 (left figure in the C panel is the grey-image scanning). (D) Protein of ABCA1 in RAW264.7 (left figure in panel D is the grey-image scanning) (* $P < 0.05$).

$p < 0.001$), while no simultaneous rise in the CPM of cholesterol ($p > 0.05$) was detected with increased IMD concentration. As for the RAW264.7, CPM was elevated as well, and a proportional rise of the concentration of IMD was observed, and was 2-fold higher in the 100 nM group (1.54 ± 0.17 vs. 0.46 ± 0.11 ,

$p < 0.001$). These results suggest that IMD is required for the promotion of the efflux of cholesterol from macrophages and exerts an anti-AS function.

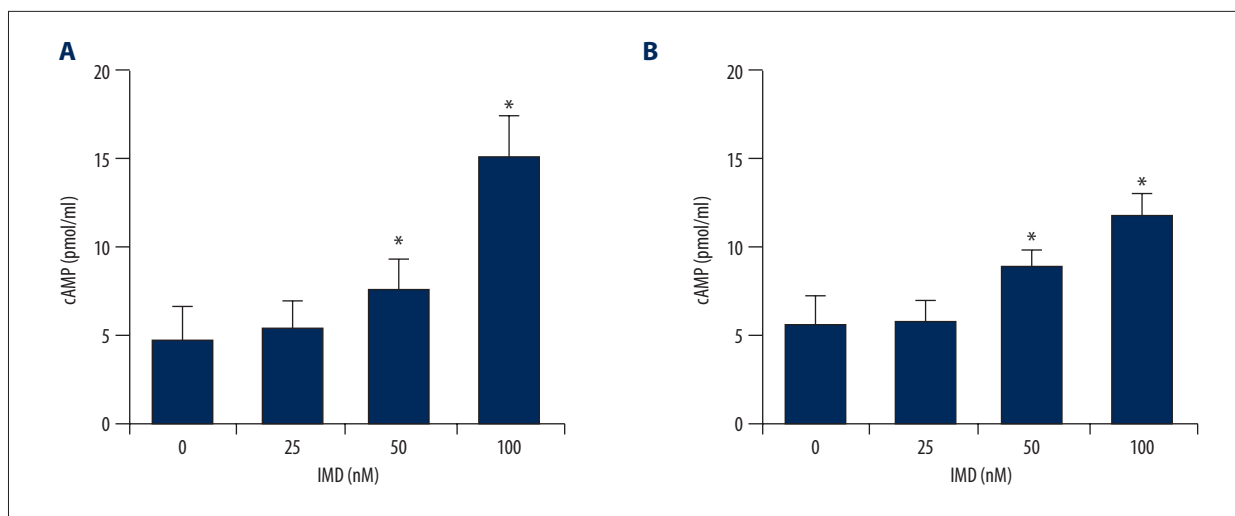


Figure 4. Intermedin enhances cAMP level in macrophages. cAMP level in transferred THP-1 and RAW264.7 was measured by ELISA after IMD infusion at 24 h. (A) cAMP level in THP1. (B) cAMP level in RAW264.7. (* $P < 0.05$).

IMD promotes the expression of ABCA1

ABCA1 is a critical mediator in the RCT process. To determine if IMD regulates cholesterol homeostasis through ABCA1, the expression of ABCA1 was detected. The mRNA of ABCA1 notably increased after IMD treatment for 24 h in THP-1 and RAW264.7 cells, which is 2 times higher in THP-1 than that in the control group and 3 times higher than in RAW264.7 cells (Figure 3A, 3B). Expression of ABCA1 protein was also found to be promoted, along with the elevated IMD infusion (Figure 3C, 3D). These findings show that IMD exerts a positive effect on ABCA1 expression, which further influenced cholesterol efflux in macrophages.

IMD enhances cAMP level in the macrophage

Since IMD affected the promotion of ABCA1, we investigated whether IMD is essential in the cAMP-PKA-ABCA1 signal pathway. ELISA was used to measure the cAMP level in transferred THP-1 and RAW264.7 after 24 h by IMD infusion. There was no significant difference in 25 nM administration of IMD for 24 h; however, when the high dose of IMD was given, cAMP rose by 38.7% in the 50-nM group and was 3 times higher in the 100-nM group compared to the control group (Figure 4A). The level of cAMP in RAW264.7 followed a similar trend as with THP-1, while cAMP was enhanced by 55.8% more than in the control group and was 100 nM higher with IMD (Figure 4B). These results show that IMD can increase the cAMP level in both of these cell lines in a dose-dependent manner, and the effect of this phenomenon was more obvious in THP-1.

PKAI inhibits ABCA1 and cholesterol efflux

To determine whether the effects of IMD on the cholesterol efflux were mediated by the cAMP-PKA-ABCA1 pathway, PKAI, a

potent inhibitor of PKA, was utilized. The expression of ABCA1 in THP-1 did not show a significant decrease with PKAI treatment. However, an interesting finding was noted with respect to RAW264.7, wherein a decreasing trend was also noted in the ABCA1 expression and cholesterol efflux (Figures 5, 6). These findings show that IMD exerts a positive effect on cholesterol efflux from the macrophages through the cAMP-PKA-ABCA1 mechanism in RAW264.7 but not in THP-1.

Discussion

The present study demonstrates that intravenous IMD infusion ameliorates AS plaque formation, decreases the level of TC and LDL, and increases HDL *in vivo*. Further, exogenous IMD administration enhances the cholesterol efflux in THP-1 and RAW264.7. Additionally, this study described a novel mechanism illustrating the effects of IMD on regulating cholesterol homeostasis via the cAMP-PKA-ABCA1 signaling pathway in RAW264.7, suggesting ABCA1 is required for the IMD-mediated cholesterol efflux.

Cholesterol efflux is a critical part of RCT to remove the overloaded cholesterol from the macrophages in the subintima of the vessel wall to HDL or apolipoprotein (apo) A-I by a variety of mechanisms, including a pathway dependent on a cell membrane protein called ABCA1. ABCA1 is a member in a large family of ABC transporters, which is characterized by two ATP binding domains and two 6-helix transmembrane domains [14]. Mutation in ABCA1 causes a severe shortage of HDL, resulting in a prevalent AS [15–17]. In the present study, we showed that ABCA1 expression in macrophages is highly regulated on both transcriptional and post-transcriptional levels by IMD treatment in THP-1 and RAW264.7 [18]. The elevated ABCA1

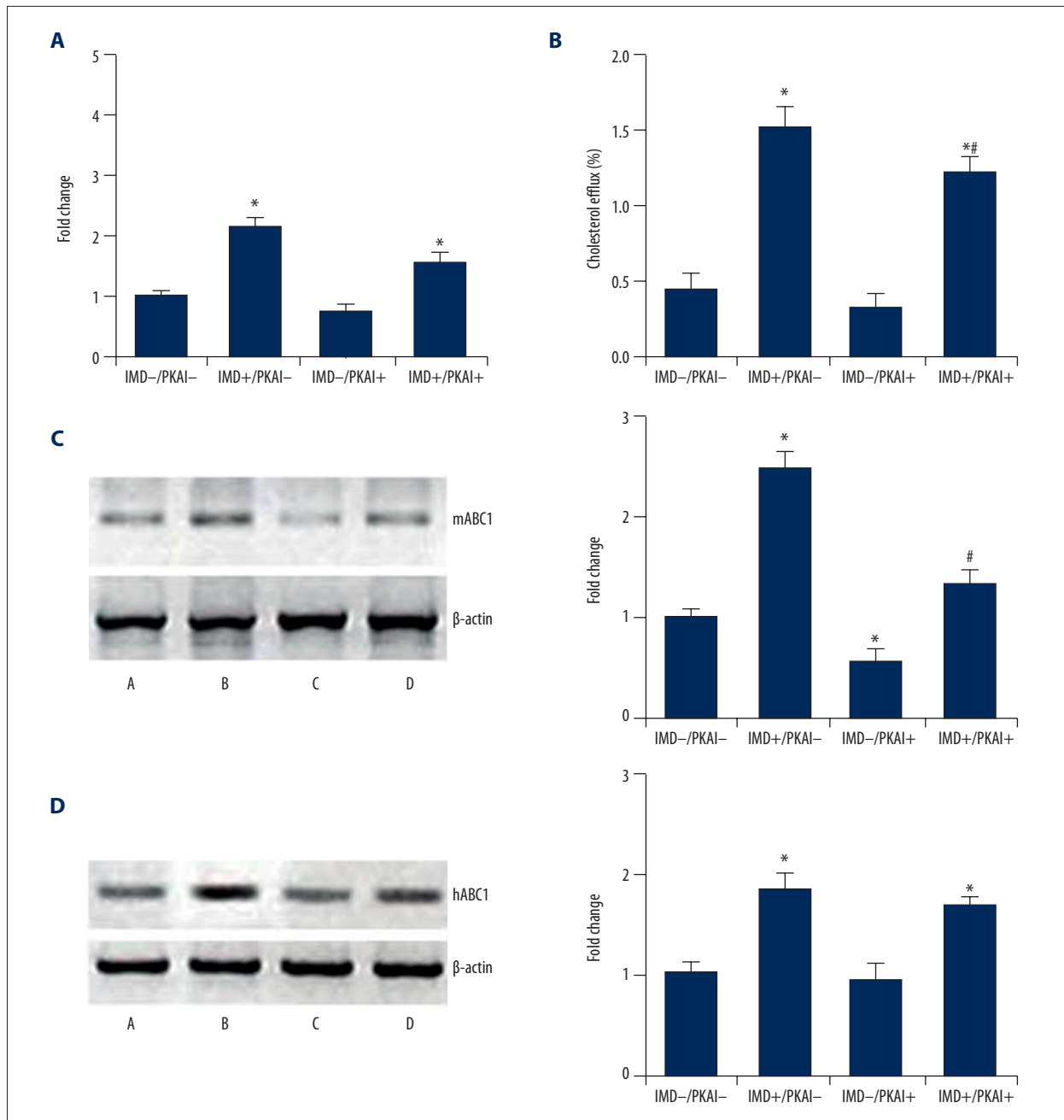


Figure 5. PKAI inhibits ABCA1 expression. Cell mRNA and protein of ABCA1 was extracted and measured by PCR and Western blot after being treated by PKAI. **(A)** mRNA of ABCA1 in THP1. **(B)** mRNA of ABCA1 in RAW264.7. **(C)** protein of ABCA1 in THP1(right figure in the **C** panel is the grey image scanning). **(D)** Protein of ABCA1 in RAW264.7 (right figure in panel **D** is the grey-image scanning) (* $P < 0.05$).

might explain the reduced serum TC, LDL-C level, and attenuated AS plaque area, and elevated serum HDL-C in ApoE-/- mice that promoted the cholesterol efflux rate from THP-1 and RAW264.7 macrophage-derived foam cells.

cAMP is a known second messenger, acting both at the mRNA and protein level, up-regulating ABCA1 expression [19,20]. The

identification of a cAMP-responsive element is considered to be important for the induction of ABCA1 gene expression. In the present study, an elevated level of cAMP in both human and mouse macrophage cell lines was observed, with promotion in THP-1 ABCA1 expression observed twice, and 3 times in RAW264.7 by IMD treatment. The present study results are in line with results reported by Hu et al., who concluded that

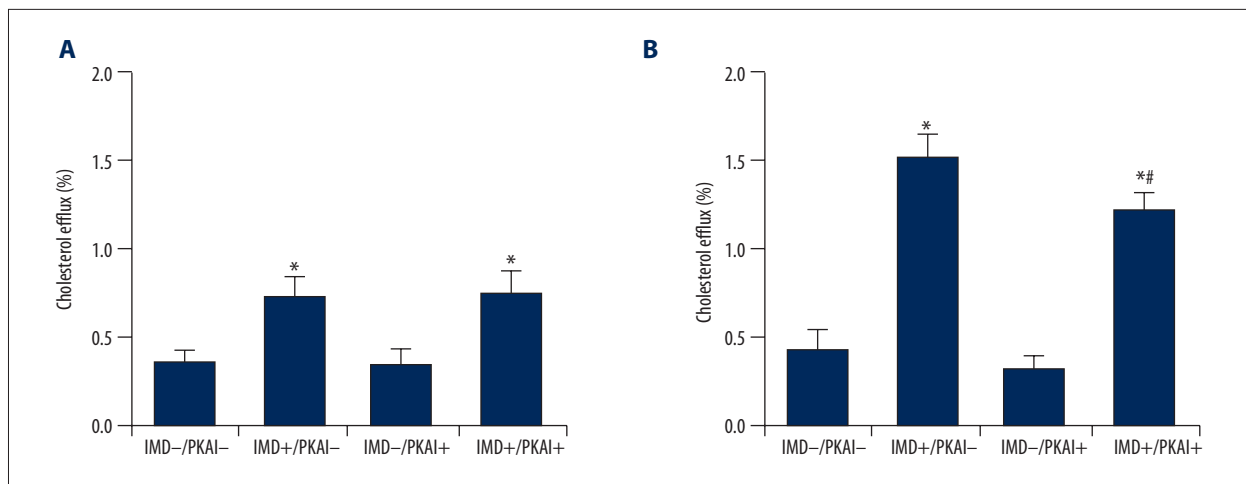


Figure 6. PKAI represses cholesterol efflux. Counts per minute (CPM) was used to measure medium and cell-associated $[^3H]$ cholesterol after PKAI treatment in the macrophages. (A) CPM in THP-1, (B) CPM in RAW264.7 (* $P < 0.05$).

eicosapentaenoic acid (EPA) inhibit the ABCA1 protein and cholesterol efflux through cAMP-PKA in THP-1 [9]. Apart from the induction of ABCA1, cAMP is also an activator of PKA, which is reported to be involved in stimulating the efflux of cholesterol. Blocking the effect of PKA has an inhibitory effect on the phosphorylation of ABCA1 and cholesterol efflux [21,22]. In the present study, we showed that PKAI obviously decreased ABCA1 expression and suppressed cholesterol efflux by IMD in RAW264.7 but not in THP-1, indicating that IMD regulates ABCA1-mediated cholesterol efflux through PKA induction in the mouse macrophages but not in the human macrophages. Our present results agree with those of several other studies, but conflict with the results reported by Hu et al., who found that ABCA1 was modulated by cAMP-PKA in THP-1, a human macrophage cell line [23]. However, Bortnick et al. earlier put forward a concept that the response to cAMP was different in human and mouse ABCA1 genes, and only the latter showed a substantial induction in macrophages by cAMP treatment [24]. It has been reported that the liver X receptors (LXR)/retinoid X receptor (RXR) pathways transcriptionally regulated ABCA1 as well, which may provide a new insight that the RXR pathway that may be involved in human macrophage cells because THP-1 lacks the cAMP response element binding protein (CREBP). Further studies in this area are needed.

IMD is a novel vasoactive peptide of the CGRP superfamily, which inhibits vascular calcification and impairs AS lesions in ApoE $^{-/-}$ mice. In recent studies, IMD was reported to ameliorate foam-cell formation via suppressing scavenger receptor A (SR-A), induced mostly by acetylated LDL (acLDL) and CD36

mainly derived from oxidized LDL (oxLDL) with IMD infusion, which both contributed to foam-cell formation [13,25]. Our study shows a novel mechanism by which IMD may be a potential therapeutic candidate for treating AS through promoting the cholesterol efflux. IMD is also a potent vasodilator by increasing cAMP production, and continuous IMD treatment decreases blood pressure and improves hemodynamic function in spontaneously hypertensive rats via the cAMP-PKA pathway [10,11,26,27].

Conclusions

In conclusion, we demonstrated a new mechanism by which IMD ameliorates the formation of foam cells and can be used to treat AS by promoting cholesterol efflux via (at least partly) a cAMP-PKA pathway-dependent mechanism in RAW264.7 and THP-1 cell lines. More specific functions and mechanisms of IMD in atherogenesis remain to be explored.

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Conflict of interests

None.

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