

Effect of adiponectin on macrophage reverse cholesterol transport in adiponectin-/- mice and its mechanism

YUERU WANG^{1,2}, XIN WANG², YINGYING GUO^{1,2},
YUNFEI BIAN^{2,3}, RUI BAI², BIN LIANG^{2,3} and CHUANSHI XIAO^{1,2}

¹Department of Cardiology, The First Hospital of Shanxi Medical University; ²Shanxi Key Laboratory of Cardiovascular Medicine and Clinical Pharmacology; ³Department of Cardiology, The Second Hospital of Shanxi Medical University, Taiyuan, Shanxi 030001, P.R. China

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Abstract. The objective of the present study was to investigate the effect of adiponectin (APN) on macrophage reverse cholesterol transport (RCT) in adiponectin-/- knockout mice (APN-/-mice) and its possible anti-atherosclerotic mechanism. A total of 30 male APN-/-mice were randomly divided into the control group and four intervention groups. The intervention groups were treated with intraperitoneal injections of APN, at doses of 50, 150, 200 and 250 $\mu\text{g}/(\text{kg}/\text{day})$, respectively, for 4 weeks. The control group received normal saline. After 4 weeks, serum lipid levels were measured, the degree of severity of atherosclerotic lesions was observed by light microscopy, the ³H-TC (APN-/-mice treated with intraperitoneal injections of ³H-TC-labeled macrophages) radioactivity in serum, liver, and feces, and the expression of ABCA1 mRNA and protein in liver were determined. Compared with the control group, serum triglycerides, total cholesterol, and low-density lipoproteins levels in the intervention groups were significantly decreased, while high-density lipoprotein was increased. The severity of aortic atherosclerotic lesions in the intervention groups was milder than in the control group, which had obvious aortic atherosclerotic lesions, large lipid deposition on vessel walls, and the formation of atheromatous plaques. In the intervention groups, serum ³H-TC content was significantly decreased ($P < 0.05$), but the ³H-TC content in liver and feces was significantly increased ($P < 0.05$). The

levels of ABCA1 mRNA in liver of the intervention groups were significantly increased in a dose-dependent manner. In conclusion, APN can promote RCT and intracellular cholesterol efflux by upregulating the expression of ABCA1, to delay the occurrence and development of atherosclerosis.

Introduction

Cardiovascular disease is currently among the most common causes of morbidity and mortality in the world, and severely threatens human health (1). Data show that there are currently roughly 230 million people suffering from cardiovascular disease in China, of whom 3 million people die every year (2). Atherosclerosis (AS) is the primary pathological basis of cardiovascular and cerebrovascular disease, in which disorders of lipid metabolism, especially cholesterol metabolism, are proven to be the main pathogenic factor. The most important pathological feature of AS is the presence of macrophages that engulf large amounts of lipid to form foam cells. Therefore, research on the pathogenesis, prevention, and treatment of AS has become an area of intense interest (3).

Under normal conditions, the intake, synthesis, and discharge of substances are in dynamic equilibrium. The dynamic balance of cholesterol is key to maintaining the cellular membrane system and the basic activities of cells. Reverse cholesterol transport (RCT) refers to free intracellular cholesterol that was transported to the outside of the cell, transported to the liver, and excreted (4). The process of RCT is as follows: under the mediation of ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette sub-family G member 1 (ABCG1), and other transport proteins, excess cholesterol in peripheral cells combines with apolipoprotein (apo) to form mature high-density lipoprotein (HDL), which is transported to the liver via blood, and excreted (5). As the largest known family of membrane transport proteins, ABCs can be divided into eight sub-families. ABCA-ABCH performs substrate transport using ATP as an energy source in cells. ABCA1, which has been widely studied, is an important protein involved in the transport process, and can also mediate intracellular cholesterol efflux. Phospholipid efflux is initiated by the ABCA1 transport protein, and in addition to apoA-I, which forms phospholipid-apoA-I compounds, jointly promote

Correspondence to: Dr Bin Liang, Department of Cardiology, The Second Hospital of Shanxi Medical University, 382 Wuyi Road, Taiyuan, Shanxi 030001, P.R. China
E-mail: liangbin0921@sina.com

Professor Chuanshi Xiao, Department of Cardiology, The First Hospital of Shanxi Medical University, 85 Jiefang South Road, Taiyuan, Shanxi 030001, P.R. China
E-mail: 237634097@qq.com

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the outflow of intracellular cholesterol. Cholesterol efflux is caught by the discoid phospholipid-apoA-I compound and esterification of extracellular free cholesterol leads to formation of mature HDL. Following blood transportation to the liver and uptake, it is transformed into bile salt and excreted in feces (6-8). Therefore, increase of RCT is of great significance for the prevention and treatment of AS.

Many studies (9,10) have shown that adiponectin (APN) secreted by fat cells plays important roles in insulin resistance (IR), anti-inflammation, and anti-AS. In animal models, decrease of APN can aggravate inflammation, thus promoting the ischemic injury of blood vessels. In patients with obesity, diabetes, hypertension, myocardial injury, and AS, APN levels are significantly decreased. Additionally, multiple clinical observations indicate that low serum APN levels are related to vascular endothelial injury, hypertension, myocardial infarction, and coronary heart disease (11). In the process of AS, the proliferation of vascular smooth muscle cells and the transformation of macrophages to foam cells can be inhibited by APN, which represents its anti-atherosclerotic role in endothelial cells (12,13).

In this study, adiponectin knockout mice (APN^{-/-} mice) fed a high-fat diet for 8 months were used to observe the pathological changes of the aortic root, and establish the model of AS. Mice in the experimental groups were treated with different doses of APN, and the changes of serum lipid levels in each group of mice were measured. The animals were injected with ³H-TC, and the cholesterol efflux in serum, liver, and feces of mice treated with different doses of APN was measured by liquid scintillation counting. The effects of APN on mRNA transcription and protein expression of ABCA1 were measured by PCR and western blot analysis respectively, to investigate the effect of APN on RCT and its possible mechanism, and to provide a new experimental basis for the prevention of AS.

Materials and methods

Reagents. APN (Biovendor GmbH, Kassel, Germany), TRIzol (Takara Bio, Inc., Otsu, Japan), PageRuler Prestained Protein Ladder, Color Pre-stained Super-high Molecular Weight Protein Marker (Thermo Fisher Scientific, Waltham, MA, USA), ABCA1 antibody, β -actin antibody (Abcam, Cambridge, UK), ³H-TC (Life Sciences, Farmingdale, NY, USA), Ultraviolet Spectrophotometer CARY50 (Varian Medical Systems, Inc., Palo Alto, CA, USA), and Microplate Reader (Autos, Sydney, Australia).

Experimental animals. Healthy 4-week-old male APN^{-/-} mice, weighing 20-25 g, were provided by Shanghai Southern Model Biotechnology Development Co., Ltd. (Shanghai, China).

Animal grouping. Healthy 4-week-old male APN^{-/-} mice were randomly divided into five groups (six in each group) after high fat feeding: i) Control group: Daily intraperitoneal injections of 0.2 ml normal saline for a total of 4 weeks; ii) APN 50 μ g/(kg/day) intervention group: daily intraperitoneal injections of 50 μ g/(kg/day) APN, for a total of 4 weeks; iii) APN 150 μ g/(kg/day) intervention group: Daily intraperitoneal injections of 150 μ g/(kg/day) APN, for a total of 4 weeks;

iv) APN 200 μ g/(kg/day) intervention group: Daily intraperitoneal injections of 200 μ g/(kg/day) APN, for a total of 4 weeks; and v) APN 250 μ g/(kg/day) intervention group: daily intraperitoneal injections of 250 μ g/(kg/day) APN, for a total of 4 weeks. This study was approved by the Animal Ethics Committee of Shanxi Medical University Animal Center.

Sample collection. After mice were fixed by perfusion, orbital venous blood was collected to isolate serum. After blood collection, the thoracic cavity was opened, and the thoracic aorta was harvested to prepare pathological sections. The liver tissues were harvested and dried with filter paper, preserved in liquid nitrogen, and the average weight was confirmed as 1.96 \pm 0.08 g. The feces were collected and weighed. The liver tissues were used to measure the levels of the ABCA1 gene.

Measurement of serum lipid level. After blood was collected, it was centrifuged at 1,000 x g for 15 min. The levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and HDL cholesterol (HDL-C) were measured by enzymic methods.

Histopathological examination of the thoracic aorta. Myocardial tissues were fixed in 4% formalin for 24 h and embedded in paraffin, sectioned into 2- μ m slices, stained by hematoxylin and eosin, and observed under a light microscope (BX-42; Olympus, Tokyo, Japan). Histological lesions were assessed as the degree of severity of atherosclerotic lesions in blood vessels.

Measurement of the rate of cholesterol efflux. The total ³H-TC radioactivity in blood was calculated, and its percentage, accounting for total radiation was calculated. Weighed liver was placed in the glass homogenizer and 20 μ l/mg hexane and isopropyl alcohol extractant was added for centrifugation. Ethanol (50%) was added to feces, dissolved to 114 g/l, and the percentage of radioactivity was calculated.

RT-PCR-mediated measurement of ABCA1 mRNA. Total RNA from liver tissues was extracted using TRIzol, and reverse transcribed into cDNA by a one-step method according to kit instructions. Next, PCR amplification was carried out. The ABCA1 primers were from Shanghai GenePharma Co., Ltd. (Shanghai, China). The thermal profile was as follows: pre-denaturation at 94°C for 5 min, 94°C for 50 sec, 56°C for 1 min, and 72°C for 1 min, for a total of 30 cycles, and 72°C for 10 min. A total volume of 2 μ l of amplification product was used for identification by agarose gel electrophoresis, and a gel imaging instrument (ChampChemTM basic; Beijing Sage Creation Science Co., Ltd.) was used for acquiring photographs and analysis.

Western blot analysis for measurement of ABCA1 protein level in liver tissue. Protein from liver tissue was extracted according to standard methods. The BCA method was used for protein quantification. Protein was separated by 10% polyacrylamide gel electrophoresis and transferred to PVDF membranes by the semi-dry method. Membranes were blocked in TBST containing 5% skim milk powder at room temperature for 3 h. Membranes were then incubated with ABCA1 primary

Table I. Comparisons of serum lipid levels among groups (mean \pm SD).

Group	TG (mmol/l)	TC (mmol/l)	HDL (mmol/l)	LDL (mmol/l)
Control	1.41 \pm 0.07	4.57 \pm 0.26	0.19 \pm 0.02	1.58 \pm 0.09
APN 50 μ g/(kg/day) intervention	1.30 \pm 0.04 ^a	4.23 \pm 0.12 ^a	0.24 \pm 0.02 ^a	1.23 \pm 0.15 ^a
APN 150 μ g/(kg/day) intervention	1.18 \pm 0.09 ^b	3.40 \pm 0.27 ^{a,b}	0.28 \pm 0.03 ^{a,b}	1.12 \pm 0.07 ^{a,b}
APN 200 μ g/(kg/day) intervention	1.08 \pm 0.09 ^{a-c}	3.25 \pm 0.21 ^{a-c}	0.38 \pm 0.02 ^{a-c}	0.90 \pm 0.05 ^{a,b}
APN 250 μ g/(kg/day) intervention	0.96 \pm 0.09 ^{a-d}	2.88 \pm 0.14 ^{a-d}	0.51 \pm 0.04 ^{a-d}	0.80 \pm 0.03 ^{a-d}

Compared with the control group, ^aP<0.05; ^bP<0.01. SD, standard deviation; APN, adiponectin; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein. ^aCompared with control, P<0.05; ^bcompared with APN 50 μ g/(kg/day), P<0.05; ^ccompared with APN 150 μ g/(kg/day), P<0.05; ^dcompared with APN 200 μ g/(kg/day), P<0.05.

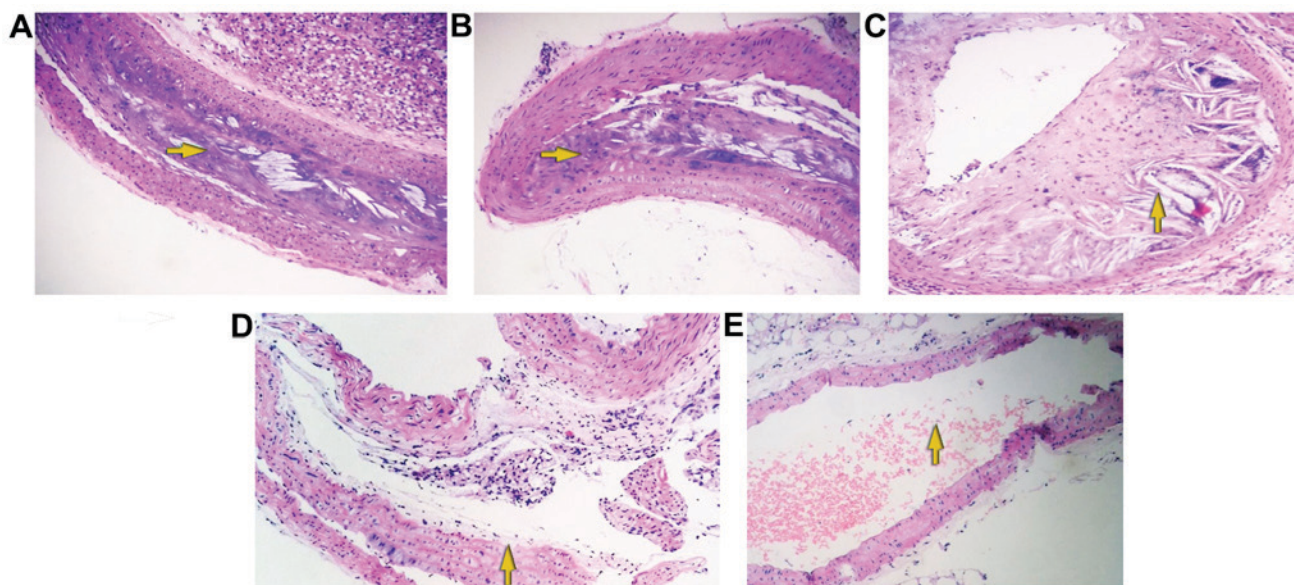


Figure 1. Representative pathological sections of the aortic root of mice in different groups (magnification, x200). (A) Control group; (B) APN 50 μ g/(kg/day) intervention group; (C) APN 150 μ g/(kg/day) intervention group; (D) APN 200 μ g/(kg/day) intervention group; (E) APN μ g/(kg/day) intervention group. APN, adiponectin. Yellow arrows, lipid deposition and formation of atheromatous plaques.

antibody (1:1,000) at 4°C for 12 h. Next, membranes were incubated in goat anti-mouse secondary antibody (1:2,000) at 4°C for 1.5 h. Finally, a gel imaging instrument was used for observation and analysis.

Statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Data are presented as mean \pm standard deviation. The single factor analysis of variance was used for comparisons between groups, and LSD-t-test was adopted for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Measurement of serum lipid levels in mice. After high fat feeding for 8 months, compared with the experimental groups that were treated with different doses of APN, in the control group, TC, TG, and LDL were significantly increased, while HDL was decreased (P<0.05). In the intervention groups, serum TC, TG, and LDL were decreased with increasing dose of APN, while HDL was increased (Table I).

Pathological changes of the aortic root in mice. Regarding APN^{-/-} mice in the control group, light microscopy demonstrated large lipid deposition on the aortic vessel wall and the formation of atheromatous plaques in the vascular lumen, with fibrous caps on the surface. Under the surface, there were scattered foam cells, and in the deep layer, there were disintegration products of necrosis and lipid deposition, which were secondary changes. When the experimental groups were treated with intraperitoneal injections of APN, the endovascular lesions were alleviated (Fig. 1).

Effect of APN on cholesterol efflux: Comparisons of ³H-TC excretion among groups. The data obtained by liquid scintillation counting showed that compared with the control group, the cholesterol efflux rate in the experimental groups increased with increasing dose of APN, and the differences were statistically significant (P<0.05; Table II).

Effect of APN on ABCA1 mRNA expression. The RT-PCR results showed that compared with the control group, the transcriptional level of ABCA1 mRNA in the experimental groups

Table II. Comparisons of ^3H -TC content among groups (mean \pm SD, cpm%).

Groups	Serum ^3H -TC	Liver ^3H -TC	Feces ^3H -TC
Control	2.58 \pm 0.13	0.56 \pm 0.02	0.62 \pm 0.02
APN 50 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	2.12 \pm 0.10 ^a	0.8 \pm 0.04 ^a	0.84 \pm 0.04 ^a
APN 150 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	1.95 \pm 0.09 ^b	1.23 \pm 0.05	1.39 \pm 0.05
APN 200 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	1.83 \pm 0.07	1.46 \pm 0.07	1.58 \pm 0.07
APN 250 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	1.66 \pm 0.05	1.57 \pm 0.09	1.66 \pm 0.09

Compared with the control group, ^aP<0.05; ^bP<0.01. APN, adiponectin.

Table III. Expression levels of ABCA1 mRNA of each group of mice (mean \pm SD).

Groups	Amount	mRNA
Control	6	1
50 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	6	1.282 \pm 0.006
150 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	6	1.785 \pm 0.008
200 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	6	2.831 \pm 0.055
250 $\mu\text{g}/(\text{kg}/\text{day})$ intervention	6	3.809 \pm 0.012

Compared with the control group, P<0.01. SD, standard deviation.

was significantly increased, and increased with increasing dose of APN, with statistically significant differences (P<0.05; Table III).

Effect of APN on the expression of ABCA1 protein. The results of western blot analysis showed that compared with the control group, the expression level of ABCA1 protein in the experimental groups was significantly increased, and increased with increasing dose of APN, with statistically significant differences (P<0.05; Fig. 2).

Discussion

In recent years, cardiovascular disease has shown a high incidence and a trend of occurring in younger patients, in whom AS is the most common disease threatening human health (14). AS is the main pathological basis of cardiovascular and cerebrovascular disease, in which disorders of lipid metabolism, especially cholesterol metabolism, are proven to be the main pathogenic factor.

Under normal conditions, the intake, synthesis, and discharge of substances are in dynamic equilibrium, which is key for maintaining homeostasis. RCT is the only method for the body to discharge excess cholesterol, which involves the transportation of excess cholesterol from peripheral cells to the outside of cells, via the action of ABCA1, ABCG1, and other transport proteins. Excess cholesterol can be combined with (apoA-I to form mature HDL, which is transported to the liver via blood, and excreted (15,16). The entire process of RCT includes four aspects, such as the efflux of excess cholesterol in peripheral tissues, the esterification of HDL-C, the transfer

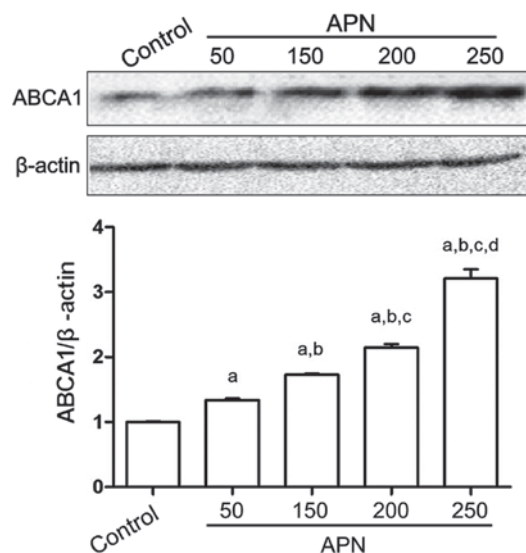


Figure 2. Expression of ABCA1 protein in control and experimental groups (treated with different doses of adiponectin): Compared with the control group, ^aP<0.05; Compared with the APN 50 $\mu\text{g}/(\text{kg}/\text{day})$ intervention group, ^bP<0.01. APN, adiponectin. ^aCompared with control, P<0.05; ^bcompared with APN 50 $\mu\text{g}/(\text{kg}/\text{day})$, P<0.05; ^ccompared with APN 150 $\mu\text{g}/(\text{kg}/\text{day})$, P<0.05; ^dcompared with APN 200 $\mu\text{g}/(\text{kg}/\text{day})$, P<0.05.

of cholesterol in HDL, and the discharge of HDL (17,18). RCT is the most important anti-atherosclerotic function of HDL-C. A large number of cholesterol receptors on the surface of mature HDL can significantly induce the cholesterol efflux, and peripheral cholesterol which was transported to the liver is ultimately transformed to bile acid and excreted, to prevent the accumulation of cholesterol in blood vessel walls (19). ABCA1 is the most important transport protein in the process of RCT, and is expressed in macrophages, T cells, B cells, and other inflammatory cells (20). ABCA1 can mediate intracellular cholesterol efflux and plays an important role in the process of RCT. ABCA1, with ATP as its source of energy, can transport excess intracellular cholesterol and phospholipids to newly generated HDL, which plays the role of an efflux pump in the process of intracellular lipid clearance, and is key for RCT. The expression of ABCA1 directly affects the RCT function of HDL-C. Increasing the expression of ABCA1 in macrophages can promote intracellular cholesterol efflux, and reduce formation of macrophage foam cells, thus preventing the formation of atherosclerotic plaques. Research on ABCA1 has identified it as a new therapeutic target for the

maintenance of lipid homeostasis *in vivo* and the prevention and treatment of AS.

LDL deposited in arterial vascular endothelium is consumed by macrophages, thus beginning the process of AS. As the transformation of macrophages to foam cells is the key process of AS, the occurrence and development of AS is therefore determined by intracellular cholesterol influx and efflux. Promoting cholesterol efflux in macrophages represents a new method for reducing the development of AS (19). The receptor for cholesterol efflux from inside to outside of the cell is HDL, while ABCA1 is the key factor for intracellular cholesterol efflux in monocytes-macrophages. As an important factor in the process of RCT, ABCA1 expression can increase in macrophages, promote intracellular cholesterol efflux, and reduce the formation of macrophage foam cells, to prevent the formation of atherosclerotic plaques.

It is well-established that adipose tissue is often regarded as an 'energy storage warehouse', which can regulate endocrine function, energy metabolism, and inflammation. Adipose tissue can secrete several hormones and cytokines, such as leptin, TNF- α , APN, and IL-6. With the improvement of living standards, the incidences of obesity, type-II diabetes mellitus (T2DM), and other disease have been increasing. In a previous study, it was shown that APN is closely related to coronary heart disease, IR, obesity, T2DM, hypertension, hyperlipidemia, and other diseases (21).

APN, which is secreted by fat cells, is a protein of 30 kDa which exists in the range of 3-30 $\mu\text{g/ml}$ in blood. APN contains 247 amino acid residues, which are divided into four regions, including the amino terminal secretion signal sequence, the small non-helical region, the collagen-like domain, and the carboxyl-terminal spherical structure domain. The plasma concentration of APN is 5-30 $\mu\text{g/ml}$, accounting for about 0.01% of total plasma protein. APN exists in three forms, the tripolymer [low molecular weight (LMW), relative molecular weight of 90,000 kDa, accounting for 25% of total APN], hexamer [medium molecular weight (MMW), relative molecular weight of 180,000 kDa, accounting for 25-35% of total APN], and polymer [high molecular weight (HMW), accounting for 40-50% of total APN] (22,23), which are not affected by circadian rhythm or diet. Compared with APN, the biological effect of globular region adiponectin (gAcrp30) is more active and extensive (24). At present, it is known that three receptors, including AdipoR1, AdipoR2, and T-cadherin are highly expressed in the cardiovascular system. AdipoR1 is widely expressed in multiple tissues and organs, showing especially high expression in skeletal muscle. AdipoR2 is mainly expressed in the liver (21). AdipoR can be expressed in islet β -cells, endothelial cells, macrophages, monocytes, and damaged vascular endothelial cells. AdipoR1 and AdipoR2 are simultaneously expressed in arterial endothelial cells and islet β -cells. The distribution of AdipoR in different tissues is distinct, while its subtypes are closely related to the binding ability and sensitivity of APN.

Recent studies found that APN can improve IR, promote anti-inflammation, improve lipid metabolism, is anti-atherosclerotic, and increases insulin sensitivity. Multiple clinical studies found that the level of serum APN is positively correlated with the age and serum level of HDL-C of patients, but is negatively related to the fasting blood glucose level,

IR index, TG, and other factors. In obesity-related diseases characterized by IR, including metabolism syndrome, diabetes mellitus, coronary heart disease, AS, and hypertension, serum APN levels exist in different degrees of low expression. APN plays an inhibitory role in the proliferation and migration of smooth muscle cells and the inflammatory response of endothelial cells by regulating lipid metabolism, and can improve endothelial cell function. APN can reduce lipid deposition by inhibiting the transformation of macrophages to foam cells, representing its protective anti-atherosclerotic role (25,26).

Previous studies found that the application of physiological doses of APN could significantly reduce the expression of cholesterol ester and lipid droplets in human monocytes-macrophages, in which the transformation of macrophages to foam cells was significantly inhibited. This suggested that APN may be a mediator between the function of macrophages and foam cells, and is also an intermediate connecting vascular inflammation and AS. Multiple studies also found that APN played an inhibitory effect in the uptake of TC by macrophages, and could significantly reduce the expression of the Class-A macrophage scavenger receptor (MSR) gene, and reduce the transformation of macrophages to foam cells. APN can increase the expression of ABCA1 mRNA and protein in foam cells, increase the content of free intracellular cholesterol, decrease the content of intracellular cholesterol ester, and significantly promote the efflux of cholesterol in macrophages, which is mediated by ABCA1. This is one of the mechanisms by which APN exerts its anti-atherosclerotic effects (27).

Recent studies indicated that serum APN levels in patients with coronary heart disease were decreased with increase of the degree of coronary AS (28). APN can modulate inflammation by regulating lipid metabolism, which is anti-atherosclerotic. In the present study, compared with the control group, the levels of TG, TC, and LDL-C in mice fed a high fat diet were increased, while HDL-C and serum APN levels were decreased. The levels of APN in mice were positively correlated with HDL and negatively related to TG (29).

Related study found that APN can upregulate the transcription and protein expression of ABCA1 in macrophages in a dose-dependent manner. Additionally, it had a significant effect on upregulation of transcription and protein expression of the ABCA1 upstream regulatory factor, LXR, which upregulates the expression of ABCA1, and increases cholesterol efflux, thus delaying the occurrence and development of AS (13).

APN can increase RCT by increasing the expression of ABCA1, to regulate lipid metabolism and reduce or delay AS. However, there are currently few studies on the effect of different doses of APN on regulating lipid metabolism and RCT in APN-/-mice.

Referring to methods previously reported in the literature (30), APN-/-mice were administered intraperitoneal injections of ^3H -TC-labeled macrophages, and the radioactivity of ^3H -TC in serum, liver, and feces was measured by *in vivo* detection to reflect RCT efficiency. The analysis by liquid scintillation counting indicated that compared with the control group, RCT efficiency was significantly increased after intervention with APN. Furthermore, the effect was

dose-dependent, confirming that APN can promote RCT in mice. PCR and western blotting showed that the mRNA transcription and protein expression of ABCA1 were significantly increased after intervention with APN, and increased with increasing dose of APN. Animal experiments confirmed that APN can promote cholesterol efflux and increase the cholesterol efflux rate by increasing mRNA transcription and protein expression of ABCA1 in a dose-dependent manner. The study confirmed that APN could promote intracellular cholesterol efflux by upregulating the expression of ABCA1, to delay the occurrence and development of AS, and provides a new experimental basis for the prevention and treatment of AS.

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