

Involvement of RBP4 in hyperinsulinism-induced vascular smooth muscle cell proliferation

Fei Li · Ke Xia · Md. Sayed Ali Sheikh ·
Jinfang Cheng · Chuanchang Li · Tianlun Yang

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Abstract Retinol-binding protein 4 (RBP4) is a newly discovered adipocytokine related to insulin resistance (IR). Hyperinsulinemia and IR are the major risk factors for cardiovascular diseases (CVD). The role of RBP4 in CVD has not yet been determined. The present study was designed to analyze the correlation of RBP4 and CVD risk factors and to evaluate the role of RBP4 in proliferation of vascular smooth muscle cells during hyperinsulinemia and the underlying mechanisms. Plasma RBP4 concentration, IR-related indexes, and cardiovascular risk factors were measured from blood samples of hyperinsulinemic rats (HIns) and control SD rats (Cons). The vascular morphology and the expression of ERK1/2, p-ERK1/2 in arterial tissues of rats were assessed. Different concentrations of RBP4 (1, 4 $\mu\text{g}/\text{ml}$) were used as intervention factor during insulin-induced aortic smooth muscle cells (RASMCs) proliferation. The expression of cell growth signaling pathways was assessed to identify the active pathway during this proliferation. Specifically, ERK1/2 inhibitor PD98059 and JAK2 inhibitor AG490 were used to detect it. RBP4 expression was higher in HIns compared with Cons

($p < 0.01$). Plasma RBP4 concentrations were positively correlated with TG ($r = 0.490$), hsCRP ($r = 0.565$), media thickness ($r = 0.890$), and p-ERK1/2 protein ($r = 0.746$) ($p < 0.05$ each). In cultured RASMCs, RBP4 enhanced insulin-induced proliferation of cells and expression of p-ERK1/2 and p-JAK2. Blockade of ERK1/2 signaling pathway inhibited RBP4-induced proliferation of RASMCs, while suppressing JAK2 remains unchanged. These results suggest that plasma RBP4 concentrations were associated with CVD. In addition, RBP4 increases the proliferation of VSMCs induced by hyperinsulinism via activation of MAPK signaling pathway.

Keywords RBP4 · Hyperinsulinism · Cardiovascular diseases · Vascular smooth muscle cell · Proliferation

Introduction

Adipose tissue is now viewed as an active endocrine organ, secreting many types of adipokines such as adiponectin, resistin, leptin, and tumor necrosis factor α [1, 2]. Retinol-binding protein 4 (RBP4), shown to be a retinol carrier protein in blood and first identified as an adipokine in 2005 using gene chips [3], is primarily secreted by the liver and adipose tissue and found to be associated with the development of IR [4–9]. In case of IR, compensatory hyperinsulinemia appears at first. It is already widely considered that hyperinsulinemia and IR are major risk factors of cardiovascular diseases (CVD), which eventually leading to the formation and development of atherosclerosis (AS) [10].

Abnormal proliferation and migration of vascular smooth muscle cell (VSMC) are responsible for AS, vascular restenosis, and other CVD. MAPK cascade is not

F. Li · K. Xia · Md. S. A. Sheikh · J. Cheng · T. Yang (✉)
Department of Cardiology, Xiangya Hospital, Central South University, Changsha 410008, China
e-mail: tianluny@163.com

K. Xia
Center for Vascular Biology and Inflammation, Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA

C. Li (✉)
Department of Geriatrics, Xiangya Hospital, Central South University, No. 87, Xiangya Road, Changsha, Hunan Province 410008, People's Republic of China
e-mail: lichuanchang@sina.com

only an important signal transduction pathway that transduces extracellular proliferation signal to the nucleus, but also the common pathway through which extracellular signals stimulate proliferation and differentiation of cells [11, 12]. Insulin activates MAPK pathway through Grb2/SOS and RAS protein to promote growth and proliferation of cell, and synthesis of collagen [13]. Hyperinsulinemia promotes insulin-induced proliferation of vascular smooth muscle cells via SHC/Raf/MAPK pathway to accelerate artery atherosclerosis [14].

As a newly discovered adipocytokine related to IR, RBP4 has been found to be very closely associated with obesity and metabolic syndrome (MetS) [4–9]. Then, about the relationship between RBP4 and CVD, there is a multiplicity of views. It has been reported that CVD risk was increased with elevated RBP4 concentrations in 1,036 patients who developed coronary artery disease (CAD) over a 6 year period, but this association was lost after adjustment for other CVD risk factors [15]. Moreover, no differences in RBP4 concentrations and RBP4-to-transferrin ratio (TTR) were observed between non-diabetic CAD patients and controls or between individuals with and without the MetS [16]. These findings suggested that serum RBP4 was not predictive of CAD risk and was not a biomarker for MetS or IR with CAD patients. In contrast, other studies showed that serum RBP4 concentration was positively correlated with carotid intima-media thickness (IMT) [17, 18], a biomarker of atherosclerosis, and was negatively correlated with flow-mediated dilatation (FMD), an expression of endothelial function. Moreover, RBP4 was found to be independently associated with low-density lipoprotein (LDL) and oxidized LDL [19, 20], suggesting that RBP4 plays an important role in atherosclerotic disorders. In elderly individuals, RBP4 concentrations were associated with MetS and in both genders but prior cerebrovascular disease in men [21], suggesting that circulating RBP4 may be a novel biomarker of metabolic complications as well as atherosclerosis and CAD. In addition, circulating RBP4 concentrations were reported to be higher in type 2 diabetic (T2DM) subjects with than without previous clinical atherosclerosis, suggesting that RBP4 may be a biomarker of CVD in T2DM subjects [22].

From the above, the role of RBP4 in CVD and IR has not yet been determined. Whether RBP4 is involved in insulin-induced proliferation of VSMCs that leading to atherosclerosis remains unclear. We, therefore, performed the present study with two related hypotheses. Firstly, we analyzed the correlation of RBP4 and CVD risk factors and vascular remodeling during hyperinsulinemia. Secondly, as proliferation of VSMC plays a vital role in AS and CVD, we evaluated the role of RBP4 in this process and the underlying signaling pathway mechanisms.

Materials and methods

Materials

Insulin injection was purchased from Wanbang biochemical medicine (10 ml: 400 μ , Jiangsu, China). RBP4 protein was obtained from Sino biological Inc. (Reconstitute at 500- μ g/mL sterile, 50 mM Tris, 10 mM CaCl_2 , and 150 mM NaCl, pH 7.5, Beijing, China). Mouse anti-extracellular signal-regulated kinase (ERK)1/2 monoclonal antibody, anti-phospho-ERK1/2 (p-ERK1/2) monoclonal antibody, rabbit polyclonal anti-JAK2 antibody, anti-phospho-JAK2 (p-JAK2) antibody, rabbit polyclonal anti-signal transducer and activator of transcription (STAT)3 antibody, and polyclonal anti-phospho-STAT3 (p-STAT3) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-rat IgG were from ZSGB-Bio (Beijing, China). Specific inhibitors of ERK1/2-PD098059 and Specific inhibitors of JAK2-AG490 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Supersignal west pico chemiluminescent substrate was bought from Thermo (Meridian, Rockford, USA).

Animal experiment

Eight-week-old male SD rats (SDs) with weight 200–250 g were obtained from animal medicine department of Xiangya Medical School, Central South University (Changsha, China). After 2 weeks adaptive feed, SDs were randomly divided into two groups: control group (Cons, $n = 7$) and hyperinsulinism group (HIns, $n = 7$). Rats in hyperinsulinism group were breed according to the previous statement [23] to make hyperinsulinism model: SDs received daily twice (9 am, 3 pm) subcutaneous injection of insulin (insulin was dissolved in acidic saline total of 0.2–10 ml of 0.1 M HCl was added to 300 ml of normal saline, then adjusted to pH 3.0). Usage and dosage of insulin: From 0.5 U/day, we increased 0.5 U gradually every day until to 6.0 U/day, then maintained this dosage to 22 days; and SDs of the control group had subcutaneous injected same volume of normal saline twice a day. Before treatment, each group of SDs had free diet (normal diet and ordinary drinking water). During the process, drinking water was changed to 5 % glucose water. Animal room's temperature was maintained to 23 ± 2 °C, while relative humidity was 45 ± 10 %. Kept quiet, dry, and ventilation indoor and changed the packing 3 times a week. All the experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication 85–23, revised 1996), and the experimental protocol was approved by the Medicine

Animal Welfare Committee of Xiangya Medical School, Central South University (Changsha, China).

Systolic blood pressure (SBP) of rats was measured by the tail-cuff method with an electro-sphygmomanometer coupled to a computerized recorder (BL-NewCentury 410 Chengdu, China) before the experiment. Each rat was kept silence in a dark and sizeable cylinder. The value for each rat was calculated as the average of three separate measurements. Blood pressure measurement was performed, in a blinded fashion, between 08:00 and 11:00 am by a single investigator.

Preparation of tissue and blood samples

The rats were anesthetized, and blood samples were collected by decapitation. The thoracic aorta was quickly removed and then divided into three parts: one part was fixed with 10 % paraformaldehyde, which paraffin sections were cut from at a thickness of 3 μm and were stained with Hematoxylineosin for evaluating vascular remodeling; and one part was homogenized by Trizol reagent (Invitrogen, Carlsbad, CA, USA) for RT-PCR analysis; the last part was homogenized in RIPA lysis buffer (Beyotime, Jiangsu, China) and 0.1 mmol/l phenylmethyl sulfonyl fluoride (PMSF) (Sigma, Missouri, USA) for immunoblotting analysis.

Biochemical assays

Blood was collected from rat tail vein for measurement of fasting plasma glucose (FPG) by Roche glucometer. Serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), LDL cholesterol (LDL-C), and high-sensitive C reactive protein (hsCRP) concentrations were measured using an autoanalyzer (Beckman CX-7 Biochemical Autoanalyzer, La Brea, CA, USA). Concentrations of fasting insulin (FIns) were assayed using a commercially-available ELISA kit (Mercodia, Sweden) in plasma frozen in $-80\text{ }^{\circ}\text{C}$, without previous thaw-freeze cycles. The average intra-assay and inter-assay coefficients of variation were 4.6 and 9.7 %, respectively. IR was estimated using HOMA-IR (Homa insulin resistance index), while HOMA-IR calculated as $[\text{fasting insulin} \times \text{fasting glucose}]/22.5$ [24].

RBP4 assay

Blood was collected from the right ventricle before the heart was arrested, and 2-ml blood was placed in a chilled tube containing enzyme inhibitors and centrifuged (within 15 min) at $4\text{ }^{\circ}\text{C}$ at 2,500 r/min for 10 min. The supernatants plasma was decanted and frozen at $-80\text{ }^{\circ}\text{C}$ until assayed. RBP4 was measured using ELISA (R&D, USA).

Intra-assay and inter-assay coefficients of variation were 5.0 and 9.8 %, respectively.

Cell culture

Rat aortic smooth muscle cells A10 (RASMCs) were obtained from ATCC (Manassas, Virginia) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 $\mu\text{g}/\text{mL}$), and NaHCO_3 (3.7 g/L), and grown in humidified atmosphere of 5 % CO_2 in air at $37\text{ }^{\circ}\text{C}$. Cells between passages 7 and 15 were used for the experiments.

Cell proliferation assays

Cell proliferation was analyzed using the MTT and cell cycle assays. For the MTT assay, RASMCs were seeded on 96-well plates at a density of $0.6\text{--}1.0 \times 10^4$ cells per well in DMEM supplemented with 10 % FBS. After 24 h, the medium was changed to DMEM containing 1 % FBS to make them quiescent for 24 h. Then, DMEM with 1 % FBS containing different concentrations of Insulin was added and co-cultured for different periods of time. DMEM with 1 % FBS containing RBP4 (1 or 4 $\mu\text{g}/\text{ml}$), or PD098059 ($5 \times 10^{-5}\text{ M}$), or AG490 ($5 \times 10^{-5}\text{ M}$) was added to pre-incubate the cells followed by treatment with Insulin (10^{-5} M). Finally, 20 μL of 5 mg/mL MTT solution was added to each well and incubated for 4 h. The supernatants were aspirated, and the formazan crystals in each well were dissolved with 150 μL DMSO. Cell proliferation was assessed by measuring the absorbance at 490 nm using a microplate reader.

For cell cycle analysis using flow cytometry, RASMCs were counted and seeded into 6-well culture plates (1×10^5 cells/well). After 24 h, the medium was changed to DMEM containing 1 % FBS to make them quiescent for 24 h. Then, RASMCs were pre-incubated with RBP4 (1 or 4 $\mu\text{g}/\text{ml}$) for 1 h, or PD098059 ($5 \times 10^{-5}\text{ M}$), AG490 ($5 \times 10^{-5}\text{ M}$) for 10 min followed by treatment with Insulin (10^{-5} M) for 24 h. After 24 h, cells were trypsinized and washed with PBS twice before fixing with ice-cold 70 % ethanol at $4\text{ }^{\circ}\text{C}$ overnight. After removing ethanol by centrifugation, cells were washed with PBS and treated with propidium iodide solution (Sigma, Missouri, USA) (50 $\mu\text{g}/\text{ml}$ propidium iodide combined with 100 $\mu\text{g}/\text{ml}$ RNase free of DNase activity, 0.1 % Triton X-100 and 0.1 mM EDTA in PBS) for 20 min in dark. After washing with PBS, propidium iodide-stained cells were subjected to cell cycle analysis using a FACS can flow cytometer (Becton–Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, at least 10,000 cells were counted. Data

Fig. 1 Characteristics and RBP4 concentrations of SDs; **a** Comparison of glycometabolism and insulin resistant between two groups; **b** Comparison of lipid metabolism and hsCRP; **c** Comparison of RBP4 concentrations. RBP4 is higher in HIns than that in Con ($p < 0.01$). Data are mean \pm SD versus con, * $p < 0.01$, # $p < 0.05$ by Student's unpaired t test. Con control group, HIns hyperinsulinism group

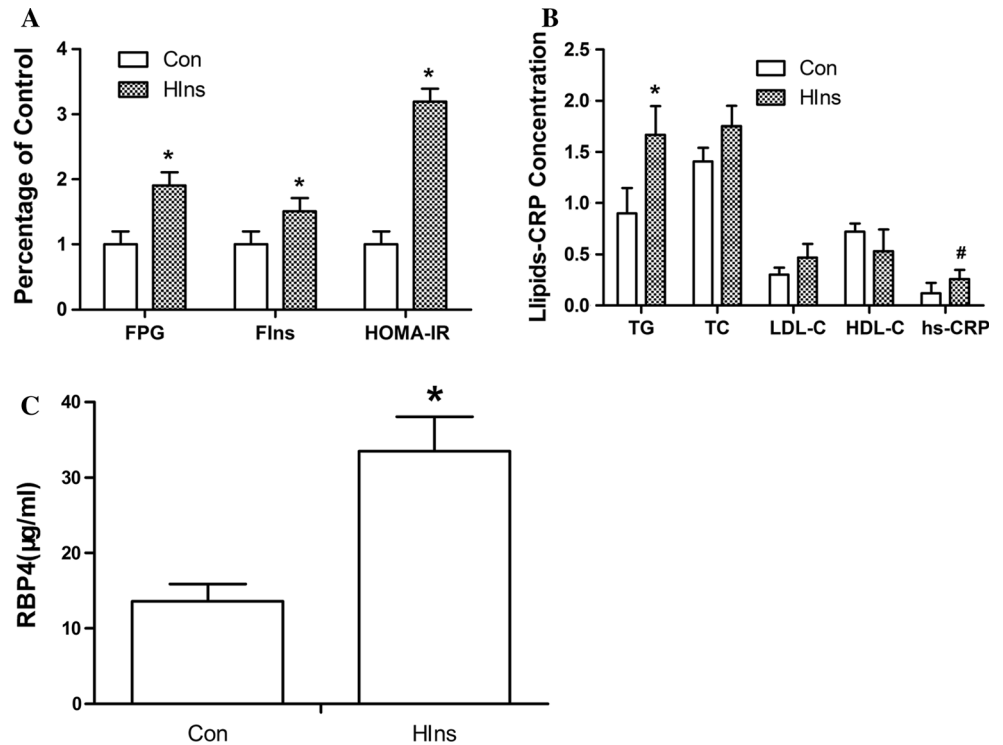


Table 1 Correlation between RBP4 concentrations and cardiovascular risk factors

	RBP4	
	r	p
Weight (g)	0.156	0.593
Systolic (mmHg)	0.119	0.418
Fasting glucose (mmol/L)	0.802	0.001
Fasting insulin (mU/L)	0.780	0.001
HOMA-IR	0.833	0.000
Triglyceride (mmol/L)	0.490	0.045
Total cholesterol (mmol/L)	0.389	0.169
LDL-C (mmol/L)	0.275	0.341
HDL-C (mmol/L)	-0.163	0.577
HsCRP (mg/L)	0.565	0.035

Coefficients (r) and p values were calculated by the Spearman rank coefficient test. The RBP4, triglycerides, HDL-C, and HOMA-IR concentrations were logarithmically transformed before statistical analysis to approximate normal distribution

are presented as the percentage of cells in a given subpopulation.

Western blotting analysis

RASMCs were lysed in RIPA lysis buffer containing 0.1 M PMSF. After centrifugation at 12,000 rpm for 20 min, the protein concentrations of the supernatants were determined with a BCA Protein Assay (Sigma; Missouri, USA). Equal

amounts of protein were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated for 5 min at 100 °C before loading. Then, equal amounts of total proteins were loaded and separated on 15 % SDS-PAGE gels at a constant voltage of 90 V for 2.5 h. Proteins were transferred onto a PVDF membrane (Millipore; Billerica, MA) using a standard transfer module for 45 min. The membrane was blocked in a 5 % dry milk/TBS-Tween 20 solution for 1 h at room temperature and then, probed with primary antibodies at a proper dilution (ERK1/2, p-ERK1/2, JAK2, p-JAK2, STAT3, p-STAT3, 1:1000; β -actin, 1:3000;) in 5 % dry milk/TBS-Tween 20 solution overnight at 4 °C. The membrane was rinsed several times with TBST-20 buffer and then incubated with HRP-conjugated second antibodies (1:5,000) for 1 h at room temperature. Excess secondary antibody was removed by three to four washes in TBST-20 buffer, and the targeted protein bands were detected using an ECL detection kit, followed by exposure of the membrane to an X-ray film. Protein bands were scanned and quantified using Image J software (version 1.40; National Institutes of Health, Bethesda, Maryland, USA). The intensities of the bands for the above indicators were normalized to those for β -actin.

Statistical analysis

Data are expressed as means \pm standard deviations (SDs) and analyzed by Student's unpaired t test for two groups or ANOVA followed by Newman-Student-Keuls test for multiple groups. Correlations between variables were

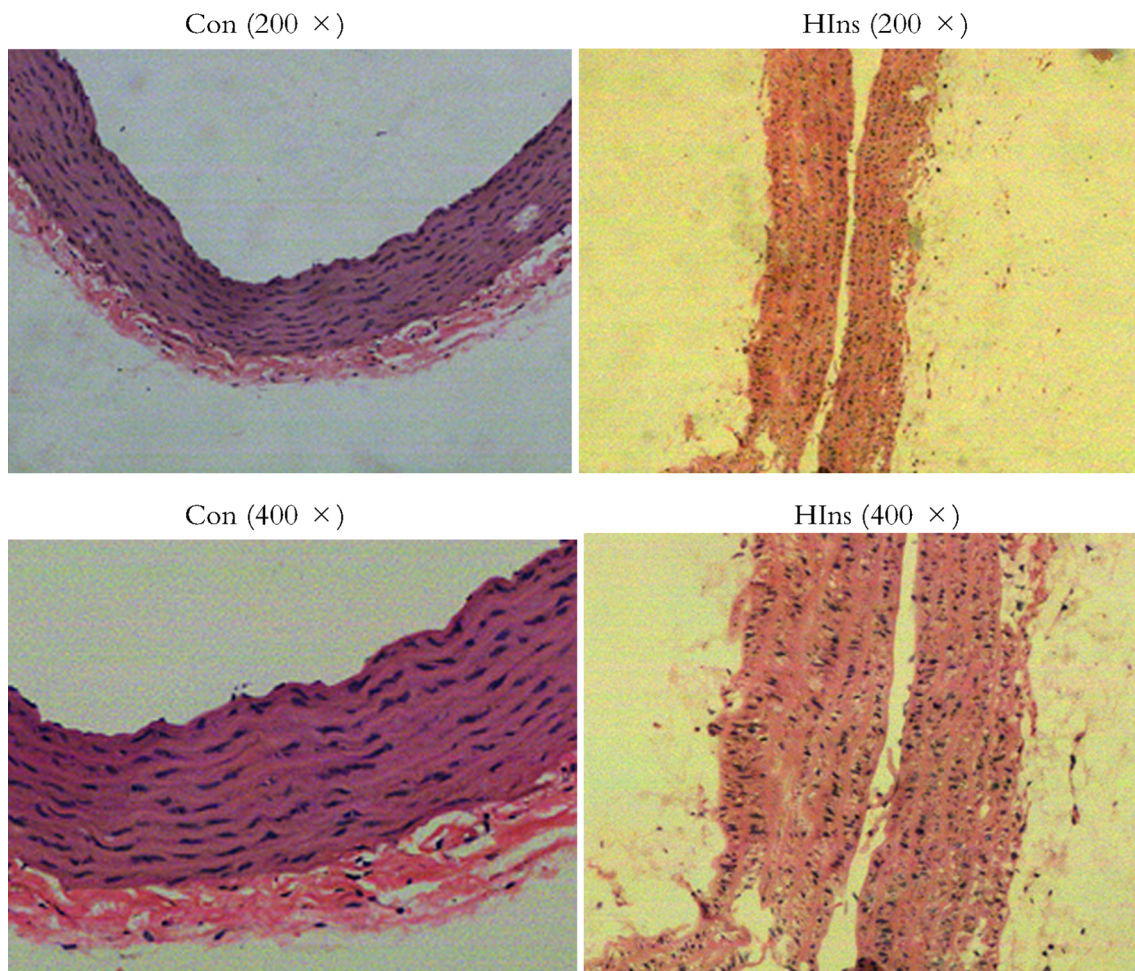


Fig. 2 Remodeling of thoracic aorta in SDs. Hematoxylineosin staining showed that in Cons group, aortic wall was not obviously thickening, and membrane of VSMC is long and thin with clear layer. In HIns group, aortic wall was thickened, endothelial cells were

injured and not continuous, endothelial gap was widened, subendocardial elastic tissue was ruptured, and the structure of VSMC media membrane was disorganized. *Con* control group, *HIns* hyperinsulinism group. *VSMC* vascular smooth muscle cell

Table 2 Correlation between RBP4 concentrations and vascular remodeling index

	RBP4	
	<i>r</i>	<i>p</i>
MT (μ m)	0.890	0.036
LD (mm)	0.787	0.070
ERK1/2 protein	0.865	0.081
p-ERK1/2 protein	0.746	0.032

Coefficients (*r*) and *p* values were calculated by the Spearman rank coefficient test. The RBP4, p-ERK1/2 protein concentrations were logarithmically transformed before statistical analysis to approximate normal distribution

calculated by the non-parametric Spearman rank coefficient test. A two-tailed *p* value less than 0.05 was considered statistically significant. Data were analyzed using SPSS 13.0 software.

Results

Characteristics and RBP4 concentrations of SDs and relationship between RBP4 and cardiovascular risk factors

There was no significant difference in the weight and systolic pressure between HIns and Cons (523.29 ± 23.3 g; 462.14 ± 55.13 g; 123.29 ± 8.3 mmHg; 119.52 ± 5.13 mmHg; $p > 0.05$). FPG, FIns, HOMA-IR, and RBP4 were significantly higher in HIns than those of Cons ($p < 0.05$ each) (Fig. 1), which suggested that our hyperinsulinemia model was successful. HsCRP and TG were higher in HIns than those in Cons ($p < 0.05$ each) (Fig. 1). While Plasma RBP4 concentrations were positively correlated with FPG ($r = 0.802$, $p = 0.001$), FIns ($r = 0.780$, $p = 0.001$), HOMA-IR ($r = 0.833$, $p = 0.000$), TG ($r = 0.490$, $p = 0.045$), and hsCRP ($r = 0.565$, $p = 0.035$).

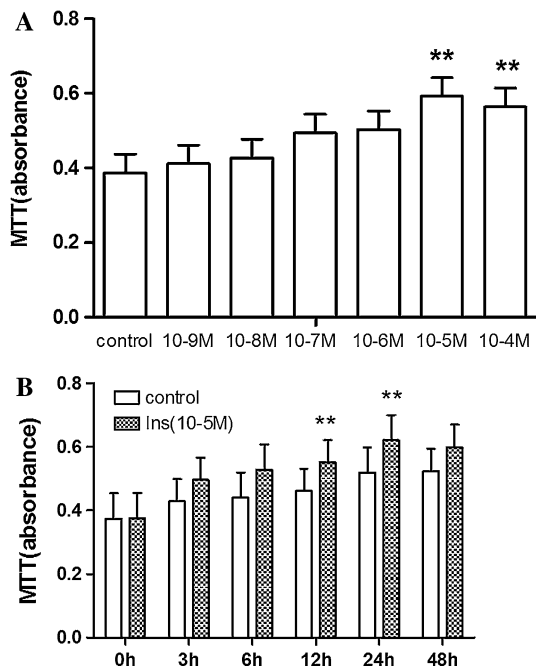


Fig. 3 Proliferation of RASMCs induced by insulin; **a** insulin induced the cell proliferation in a concentration-dependent manner; **b** insulin induced the cell proliferation in a time-dependent manner, to 24 h to the peak. The proliferation was determined using both MTT assay and flow cytometry. Data are from 8 to 10 independent experiments ($n = 8-10$). * $p < 0.05$, ** $p < 0.01$ versus Control (0 h). RASMCs Rat aortic smooth muscle cells

HOMA-IR showed the strongest correlation with RBP4 ($r = 0.833$, $p < 0.001$) (Table 1).

Plasma RBP4 concentrations and remodeling of thoracic aorta in SDs

Hematoxylineosin staining showed that in HIns group, aortic wall was thickened; the structure of VSMC media membrane was disorganized. In Cons group, aortic wall was not obviously thickening, and membrane of VSMC is long and thin with clear layer (Fig. 2). It also showed that media thickness (MT), lumen diameter (LD) of thoracic aorta in HIns were higher than those in Cons (MT: $108.00 \pm 20.05 \mu\text{m}$: $83.28 \pm 12.15 \mu\text{m}$, LD: $1.83 \pm 0.18 \text{ mm}$: $1.68 \pm 0.09 \text{ mm}$), but without statistical significance ($p > 0.05$).

As to growth signaling pathways, the expression of ERK1/2, p-ERK1/2 in the thoracic aorta were detected with Western blotting, which showed that p-ERK1/2 protein level in thoracic aorta of HIns was significantly higher than that of Cons ($p < 0.01$). While plasma RBP4 concentrations were positively correlated with MT ($r = 0.890$, $p = 0.036$), and p-ERK1/2 protein expression levels ($r = 0.746$, $p = 0.032$) (Table 2). In addition, after adjustment for insulin level, RBP4 concentrations were still positively

correlated with p-ERK1/2 protein expression ($r = 0.993$, $p = 0.007$).

RBP4 enhanced insulin-induced proliferation of RASMCs

As mentioned above, plasma concentrations of RBP4 is increased in hyperinsulinemia rats, and be positively correlated with cardiovascular risk factors including TG and hsCRP and remodeling of thoracic aorta. We, therefore, used insulin as a stimulator first in vitro VSMC experiment. In keeping with previous study [25], insulin induced proliferation of RASMCs in a concentration- and time-dependent manner as shown by the increase in formazan absorbance (Fig. 3). In addition, insulin enhanced the expression of ERK1/2, p-ERK1/2, JAK2, p-JAK2, STAT3, and p-STAT3 in a time-dependent manner as shown by the results of western blotting (Fig. 4).

To confirm the role of RBP4 in insulin-induced proliferation of VSMCs, we pre-incubated RASMCs with RBP4 for 1 h followed by treatment with Insulin for 24 h, proliferation of cells was enhanced as shown by the increase in formazan absorbance and in the proportion of cells in the S + G2 phase ($p < 0.05$, Fig. 5a, b). In addition, we found in RASMCs that expression of insulin up-regulated p-ERK, p-JAK2 were enhanced by RBP4 ($p < 0.05$ each), while p-STAT3 unchanged (Fig. 5c).

Involvement of ERK1/2, JAK2/STAT3 pathway in insulin-induced cell proliferation intervened by RBP4

To explore whether ERK1/2 or JAK2/STAT3 pathways is involved in RBP4 enhancing insulin-induced cell proliferation, PD98059 (the specific ERK1/2 inhibitor) and AG490 (the specific JAK2 inhibitor) were used. As shown in Fig. 6, pre-treatment with PD98059 ($5 \times 10^{-5} \text{ M}$) obviously inhibited proliferation of RASMCs detected by MTT and flow cytometry ($p < 0.01$) respectively. While pre-treatment with AG490 ($5 \times 10^{-5} \text{ M}$) had no effect on proliferation. PD98059 alone, but not AG490 alone inhibited RASMCs proliferation (Fig. 6).

Discussion

The main findings of the present study are as follows: (1) The plasma concentrations of RBP4 are elevated in hyperinsulinemia SDs, and have positive correlation with the cardiovascular risk factors and remodeling of thoracic aorta; (2) Insulin induced proliferation of RASMC via MAPK and JAK2/STAT3 pathway; (3) RBP4 up-regulated insulin-induced proliferation of RASMC via MAPK not

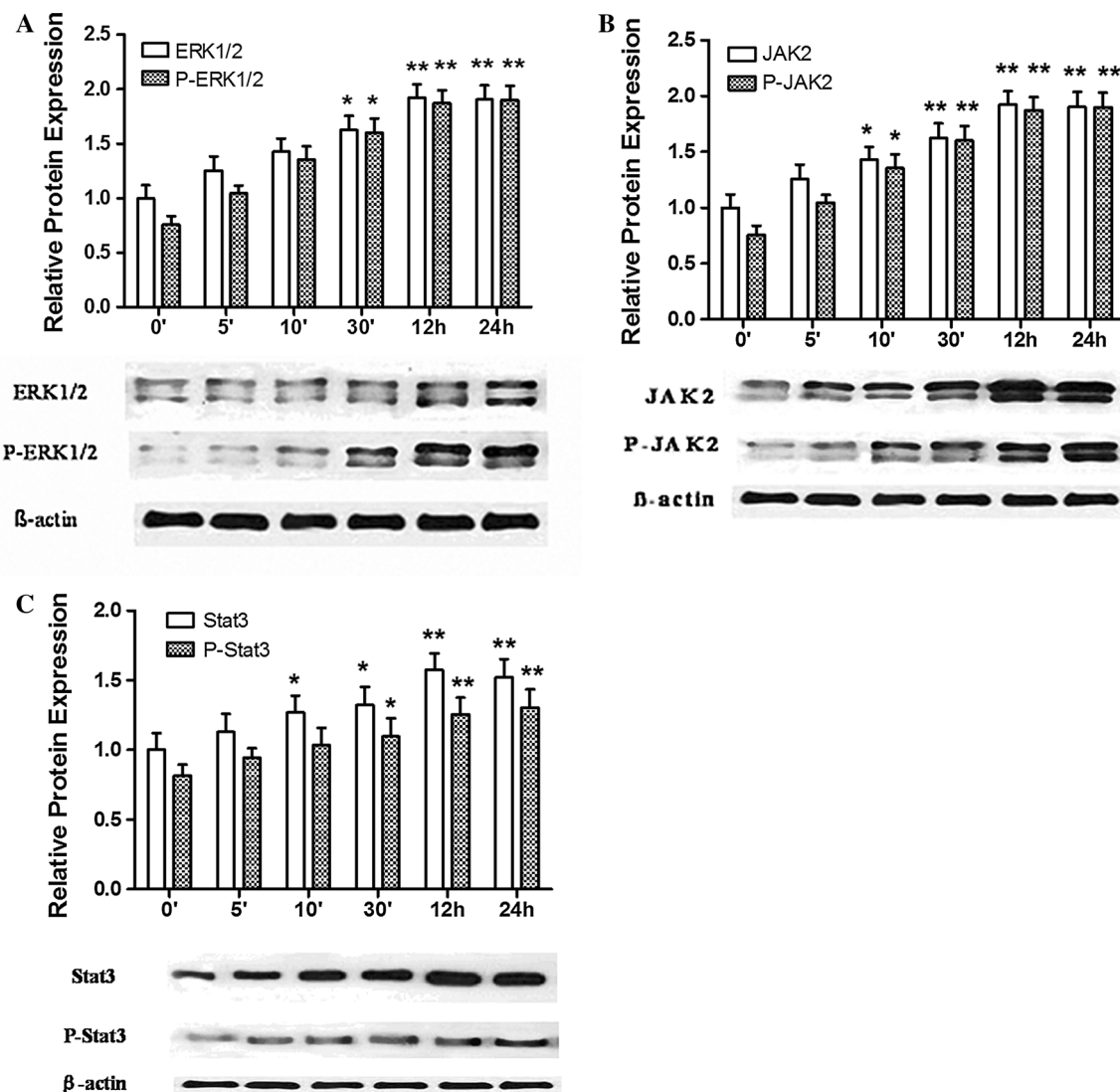


Fig. 4 Expression of ERK2, p-ERK2, JAK2, p-JAK2, STAT3, p-STAT3 in RASMCs induced by insulin; **a** Insulin induced the expression of ERK1/2, p-ERK1/2 in time-dependent manner, with the maximum amount of expression at 24 h; **b** insulin induced the expression of JAK2, p-JAK2 in time-dependent manner, with the maximum expression of JAK2 at 12 h, while P-JAK2 at 24 h;

c Insulin induced the expression of STAT3, p-STAT3 in time-dependent manner, with the maximum expression of STAT3 at 12 h, while p-STAT3 at 24 h. Data are representatives of three independent experiments ($n = 3$). * $p < 0.05$, ** $p < 0.01$ versus Control (0' without insulin). RASMCs Rat aortic smooth muscle cells

JAK2/STAT3 pathway. Collectively, these findings suggest, for the first time, that RBP4 plays an important role in the mediation of proliferation of VSMCs induced by insulin, which may contribute to vascular remodeling in hyperinsulinemia.

Since Yang et al. [3] have firstly found RBP4 in experiments with mice, related studies about RBP4 and insulin resistance, obesity, type 2 diabetes, CVD have aroused great concern. Whether RBP4 be associated with AS or CVD is still controversial. In keeping with recent study [15, 19, 26, 27], we also found Plasma RBP4 concentrations was significantly associated with independent risk factors for coronary atherosclerosis such as TG and inflammatory factors hsCRP. Verges et al. [28] found that

plasma RBP4 level was associated with plasma triglycerides, independently of liver fat content. This reinforced the idea of a robust and independent link between RBP4 and triglycerides. In addition, they showed that RBP4 was significantly and inversely associated with VLDL-apoB100 total FCR, independently of plasma triglycerides, indicating a tight association between RBP4 and VLDL catabolism. Consistently, circulating RBP4 is also found positively correlated with TC [18, 29, 30], LDL-C [6, 29, 31], triglycerides [5, 21, 29–32], and negatively correlated with high-density lipoprotein cholesterol (HDL-C) [6, 28, 30, 32]. Several studies have been able to show that the association between plasma RBP4 and triglycerides was

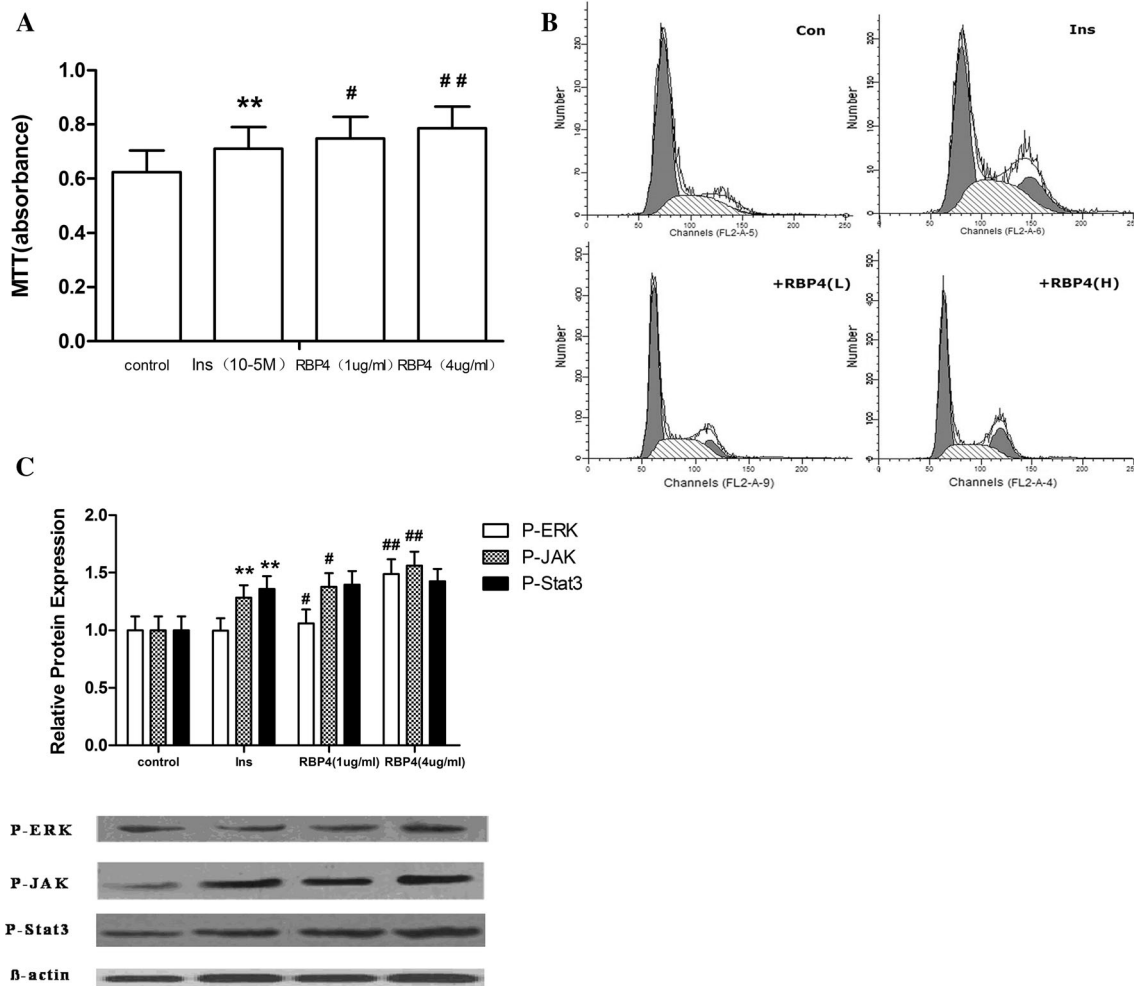


Fig. 5 RBP4 increased insulin-induced proliferation of RASMCs; **a** RBP4 increased insulin-induced cell proliferation determined using MTT assay. Data are from 8 to 10 independent experiments ($n = 8-10$) versus control $**p < 0.01$, versus Ins $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; **b** RBP4 increased insulin-induced cell proliferation determined using flow cytometry. Control: wild-type cells; Ins: wild-type cells were treated with 10^{-5} M insulin for 24 h;

+RBP4(L): cells were pre-treated with RBP4 (1 $\mu\text{g}/\text{mL}$) for 1 h before treatment with insulin (10^{-5} M) for 24 h; +RBP4(H): cells were pre-treated with RBP4 (4 $\mu\text{g}/\text{mL}$) for 1 h before treatment with insulin (10^{-5} M) for 24 h; **c** RBP4 increased insulin up-regulated P-ERK, P-JAK expression, but not P-STAT3. Data are representatives of three independent experiments ($n = 3$) versus Control $*p < 0.05$, $**p < 0.01$; versus Ins $^{\#}p < 0.05$, $^{\#\#}p < 0.01$

independent of insulin resistance [5, 9, 19, 27]. As we previously suggested [33], retinoids increase the expression of liver apolipoprotein CIII (Apo C-III), an agonist of lipoprotein lipase, through the RAR/RXR pathway. RBP4 may inhibit the catabolism of very low-density lipoprotein (VLDL), increasing VLDL levels and decreasing HDL-C. To maintain VLDL catabolism, cholesterol esterification and transfer protein pathways are activated, increasing TC and TG transport and increasing the concentrations of both in the blood. Then, a number of studies [34, 35] prompted circulating triglyceride concentrations were independent risk factors for coronary events, and abnormalities or disorders of cholesterol lipoprotein metabolic was associated with hematological disorders, such as changes in blood

coagulation and fibrinolysis. The above changes are all closely related to CVD.

In addition, it is not just a lipid deposition, but inflammation also plays an important role in the formation and rupture of atherosclerotic plaque [36]. HsCRP, as a classic acute reactive protein, has been accepted that its' increased plasma concentrations are sensitive indicators of the severity of the inflammatory. HsCRP level was elevated in local and systemic inflammatory response, and hsCRP is also an independent risk factor for cardiovascular disease [37]. In summary, the close relation between RBP4 and TG, hsCRP in the present study verifies that RBP4 occupy a seat in the process of coronary atherosclerosis, may become a new marker for coronary heart disease.

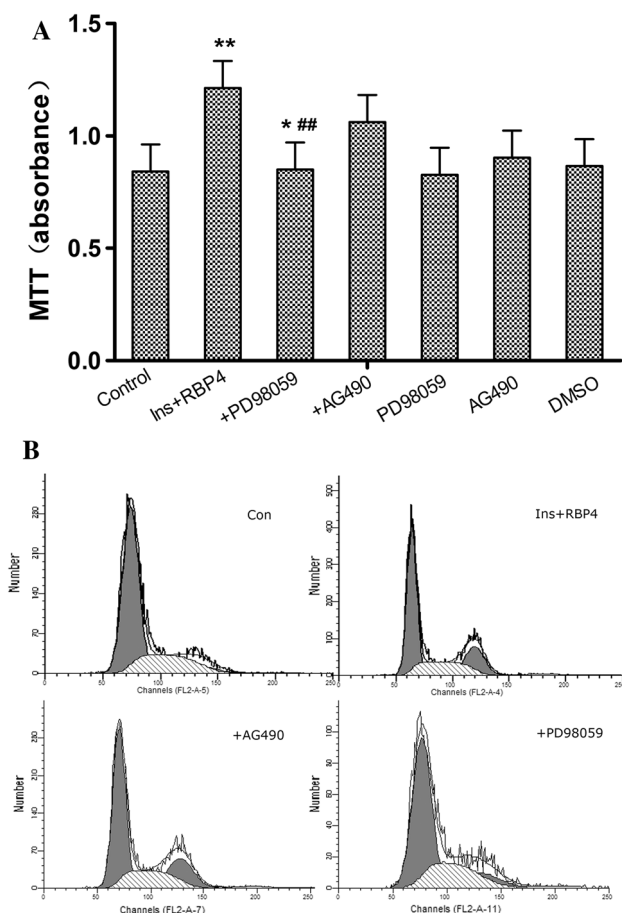


Fig. 6 Involvement of ERK1/2, JAK2/STAT3 pathway in insulin-induced cell proliferation intervened by RBP4 in RASMCs; **a** Proliferation of RASMCs measured by MTT assay. RBP4-induced proliferation of RASMCs was inhibited in the presence of the specific JAK1/2 inhibitor PD98059 (5×10^{-5} M) and not with JAK2 inhibitor AG490 (5×10^{-5} M); **b** Cell cycle analysis by flow cytometry. Control: wild-type cells; Ins + RBP4: wild-type cells were treated with 10^{-5} M Ins + RBP4 (4 μ g/mL) for 24 h; +PD98059: cells were pre-treated with PD98059 (5×10^{-5} M) for 10 min before treatment with Ins (10^{-5} M) + RBP4 (4 μ g/mL) for 24 h; +AG490: cells were pre-treated with AG490 (5×10^{-5} M) for 10 min before treatment with Ins (10^{-5} M) + RBP4 (4 μ g/mL) for 24 h; PD98059: wild-type cells were treated with PD98059 (5×10^{-5} M) for 24 h; AG490: wild-type cells were treated with AG490 (5×10^{-5} M) for 24 h; DMSO: wild-type cells were treated with 0.1 % DMSO (PD98059 isovolumetric) for 24 h. Data are from 3 to 8 independent experiments ($n = 3-8$) versus Control ** $p < 0.01$, versus Ins (10^{-5} M) + RBP4 (4 μ g/mL) # $p < 0.05$, ## $p < 0.01$. RASMCs rat aortic smooth muscle cells

Abnormal proliferation and migration of VSMC are one of the common foundations of atherosclerosis, vascular restenosis, and other vascular diseases. Furthermore, we also detected remodeling of thoracic aorta in two groups of rats, and found that media thickness (MT) and p-ERK1/2 protein expression levels were significantly positively related with RBP4. These results suggest that RBP4 and proliferation of VSMC are closely related and maybe a

regulatory factor for hyperinsulinism-induced proliferation of vascular smooth muscle.

In keeping with previous study [25], we found that insulin induced proliferation of RASMCs in a concentration-dependent manner, while in concentration of 10^{-5} M, the effect of insulin is the strongest. We also found insulin induced expression of ERK1/2 and p-ERK1/2 in a time-dependent manner, and the strongest effect was observed during 24 h. Combined with previous studies, we verify that high concentrations of insulin stimulate RASMCs proliferation through activation of MAPK. Interestingly, we also found that high concentrations of insulin showed a time-dependent stimulation of expression of JAK, p-JAK, STAT3, and p-STAT3 in RASMCs. We clearly understand that another important cell proliferation signal transduction pathway—JAK/STAT is also regulated by high-concentration insulin. JAK/STAT pathways not only mediate IL-6 [38], platelet-derived growth factor BB [39], IL-18 [40] induced proliferation of VSMC, but also have a place in hyperinsulinism-induced atherosclerosis.

As Takebayashi et al. said [41], the serum concentration of RBP4 is approximately 40–50 μ g/ml in humans [27, 42], which is approximately 10-fold the concentration used in the in vitro and ex vivo experiments in this study. However, most RBP4 is bound to retinol in the circulation, with a reported binding ratio of approximately 85 % [43]. Therefore, free (unbound) RBP4 (apo-RBP4) levels in the circulation are similar to the RBP4 concentration used in this study. In this study, RASMCs were pre-incubated with RBP4 (1 or 4 μ g/ml) for 1 h before the final concentration of 10^{-5} M Insulin, we first discovered RBP4 significantly increased the ability of insulin-induced smooth muscle cell proliferation, the ratio cells in S + G2 phase intervened by RBP4 is much larger than that of the control. The team also found that RBP4 significantly enhanced insulin-induced expression of p-ERK, p-JAK protein, meaning significantly enhanced the degree of phosphorylation of ERK and JAK, but had no significant effect on STAT3. Thereby, we speculate that RBP4 enhances insulin-induced smooth muscle cell proliferation via the MAPK pathway. Pre-treatment with ERK1/2 inhibitor PD98059 in RASMCs can significantly inhibit the above effect of RBP4, and can also significantly inhibit RBP4 induced cell cycle change. However, the JAK inhibitor AG490 treatment had no significant effect on RBP4-induced cell proliferation, while cell cycle is not obviously changed. So far, we were recommended that in the process of RBP4 promoting insulin-induced proliferation of RASMCs, MAPK pathway is activated.

As mentioned earlier, there are at least two insulin related signal transduction pathway: one is phosphatidylinositol 3 kinase (PI-3K) pathway which is activated by insulin receptor substrate protein (IRS); the other is activation of

MAPK pathway through Grb2/SOS and RAS protein. Elevated plasma RBP4 can enhance insulin resistance through inhibition of phosphorylation of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-K) in skeletal muscles [3]. The same time, studies [44] have shown that RBP4 weakened insulin-induced phosphorylation of IRS-1 and ERK1/2 in human adipose tissue. Takebayashi et al. [41] also found that RBP4 increased production of NO by strengthening PI3K/Akt/eNOS pathway in vascular endothelial cells, and led to vasodilation through inhibition of phosphorylation of ERK1/2 and insulin-induced secretion of endothelin (ET-1). However, in our study, RBP4 enhanced phosphorylation of ERK1/2 in RASMCs, thereby promoting the proliferation of smooth muscle cells, and this acceleration of RBP4 was attenuated by specific inhibitors of ERK1/2 PD98059. In target tissues and organs of obesity and type 2 diabetes, IRS/PI-3K pathway is significantly damaged, but Shc/Raf/MAPK pathway remains intact, even strengthened [14]. This “selective insulin resistance” phenomenon makes the metabolic regulation of insulin weakened, including antagonistic effect in atherosclerosis. RBP4 can inhibit phosphorylation of IRS-1 [3, 44], then promote the selective insulin resistance, to enhance insulin-induced proliferation of vascular smooth muscle cells, synthesis of collagen and promoting synthesis of growth factor.

Conclusion

In conclusion, the present study suggests that plasma RBP4 levels were associated with IR and CAD. In addition, for the first time, we find that RBP4 increases the proliferation of VSMCs induced by insulin via activation of MAPK signaling pathway and significantly regulated the atherosclerosis in hyperinsulinemia of the cardiovascular diseases. But this find is just the primary stage, further studies are required to confirm the deep and specific mechanisms.

Conflict of interest The authors declare that they have no conflict of interest.

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