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ORIGINAL ARTICLE

Hyperlipidemic plasma molecules bind and inhibit adiponectin activity

Yan-Qing Zhang[1], Sen Fan[2], Wen-Qing Wang[3], Wayne Bond Lau[4], Jian-Li Dai[2], Hai-Feng Zhang[5], Xiao-Ming Wang[2], Xiao-Gang Liu[6], Rong Li[2]*

[1]Department of Anesthesiology, School of Anesthesiology, The First Hospital, Shanxi Medical University, Taiyuan, China, [2]Department of Geriatrics, Xijing Hospital, Airforce Military Medical University, Xi'an, China, [3]Department of Hematology, Tangdu Hospital, Airforce Military Medical University, Xi'an, China, [4]Department of Emergency Medicine, Thomas Jefferson University, Philadelphia, USA, [5]Department of Teaching and Experiment Center, Airforce Military Medical University, Xi'an, China, and [6]The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, China

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***Correspondence**
Rong Li
Tel.: 86-29-84775547
Fax: 86-29-84775543
E-mail address:
wwqlrrs@fmmu.edu.cn

Xiao-Gang Liu
Tel.: 86-139 911 01973
Fax: 86-29-82665836
E-mail address:
xiaogangliu@xjtu.edu.cn

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ABSTRACT

Introduction: Adiponectin is a potent vascular protective molecule. Recent findings have suggested adiponectin resistance during early diabetes. However, the molecular mechanisms responsible remain unidentified. Here, we took an unbiased approach to identify whether hyperlipidemic plasma molecules exist that bind and inhibit adiponectin function, contributing to adiponectin resistance and diabetic vascular injury.

Methods: Adult rats were randomly assigned to receive either a normal or a high-fat diet for 8 weeks. Plasma was co-immunoprecipitated with anti-APN antibody and analyzed by mass spectrometry. The APN binding molecules and their effect upon APN biological activity were determined.

Results: As expected, the high-fat-diet increased plasma triglyceride, total cholesterol, and low-density lipoprotein. Importantly, the circulating APN level was significantly increased at this time point. Mass spectrometry identified 18 proteins with increased APN binding in hyperlipidemic plasma, among which four proteins critical in lipid metabolism, including apolipoprotein A1 (APOA1), APOA4, APOC1, and paraoxonase 1, were further investigated. Incubating recombinant APN with APOA1 markedly ($P < 0.01$), and incubating with APOC1 significantly ($P < 0.05$), inhibited APN activity as evidenced by the reduced AMPK activation in HUVECs. APOA4 and paraoxonase 1 incubation had no effect upon APN activity. Finally, plasma APOA1 was significantly increased ($P < 0.05$) in hyperlipidemic plasma compared with the control plasma.

Conclusions: It was demonstrated for the first time that increased APOA1 and APOC1 in hyperlipidemic plasma binds and inhibits APN activity. This result not only identifies a novel molecular mechanism responsible for adiponectin resistance during early stage diabetes, but also provides additional new insight into the diverse/controversial (protective and harmful) functions of high-density lipoprotein.

INTRODUCTION

Hyperlipidemia is an important clinical manifestation of metabolic syndrome. Hyperlipidemia-induced endothelial dysfunction is the pathophysiological basis of various cardiovascular and cerebrovascular complications. Understanding the pathogenesis of hyperlipidemia-induced cardiac and cerebrovascular diseases will provide insight towards the prevention and treatment of these diseases.

Adiponectin (APN) is a multifunctional protein secreted by adipocytes, and possesses numerous physiological effects such as insulin sensitization, myocardial protection, improvement of endothelial function, and anti-inflammation[1]. Studies demonstrate that hyperlipidemia in obesity/diabetes suppresses APN production, contributing to diabetic vascular injury[2,3]. However, increasing evidence exists that plasma adiponectin levels are

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increased, but the biological function is reduced, during the early stage of high-fat-diet induced diabetes, suggesting that somehow impaired adiponectin function is a new mechanism contributing to cardiovascular pathogenesis[4].

In a recent study[5], we demonstrated for the first time that plasma APN levels were significantly increased and vascular APN receptors (AdipoR1 and AdipoR2) remained unchanged 8 weeks after high-fat-diet feeding. However, phosphorylated adenosine monophosphate activated protein kinase (pAMPK) and endothelial nitric oxide synthase (eNOS) in vascular tissue were significantly reduced. Most interestingly, pre-incubation of recombinant APN with obese/hyperlipidemic plasma, but not with normal plasma, significantly reduced its AMPK and eNOS activation effect, and blunted its protective effect against tumor necrosis factor α (TNFα)-induced apoptosis of the human umbilical vein endothelial cell (HUVEC)[5]. These results suggest that serum substances exist in hyperlipidemia that inhibit APN activity. However, the identity of the hyperlipidemic plasma molecules capable of inhibiting and inhibiting APN activity remains completely unknown.

Taking an unbiased approach followed by functional determination, we attempted to identify the specific molecules in high-fat plasma that inhibit APN activity, and are therefore responsible for impaired adiponectin function.

MATERIALS AND METHODS

All experiments in this study were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals, and were approved by the Air Force Medical University Committee on Animal Care (approval number: 20150302).

Animals and cells

Twenty 4-week-old male Sprague–Dawley rats were provided by the Experimental Animal Center of the Air Force Military Medical University. Human umbilical vein endothelial cells were originally obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and kindly provided by Professor Hai-Feng Zhang of the Air Force Military Medical University. HUVECs were grown in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin and streptomycin in an incubator under standard cell culture conditions of 37°C, 5% CO_2 and 95% humidity.

Establishment of rat hyperlipidemia model

Twenty rats were randomly divided into hyperlipidemia and control groups (10 rats per group). The animals were fed a high-fat diet (78.8% basic feed, 10% lard, 10% egg yolk powder, 1% cholesterol, 0.2% pig bile salt) or a normal diet, respectively, for 8 weeks.

Measurement of plasma molecules

After 8 weeks, the rats were anesthetized with 2.5% isoflurane. Blood was sampled from the inferior vena cava. The fasting blood glucose level was detected by a blood glucometer. Plasma was isolated by centrifugation. A microplate reader (iMArk, Bio-Rad, Hercules, CA, USA) determined levels of plasma total cholesterol (TC) and triglyceride (TG) (via enzymatic method) and high-density lipoprotein (HDL)-C and LDL-C (via direct method) by use of kits provided by Jiancheng Biotechnology (Nanjing, China). The concentrations of plasma APN and its conjugates were measured by ELISA (Xitang, Shanghai, China). All detection procedures were performed according to the manufacturers' protocol for the kit.

Co-immunoprecipitation and mass spectrometry of plasma APN and its conjugates

First 500 μL plasma was mixed with 2 μL anti-APN antibody (Cat. No.: ab62551; Abcam, Cambridge, MA, USA) at 4°C overnight. Subsequently, 50 μL protein A sepharose beads (Beyotime Biotechnology, Shanghai, China) was added and gently shaken at 4°C for 4 h. After centrifugation, the precipitates were collected and washed with buffer solution for 20 min three times. After separation by SDS-PAGE electrophoresis, the APN co-immunoprecipitated proteins were subjected to liquid chromatography–tandem mass spectrometry (MS). Samples were digested in-gel with trypsin and injected onto a UPLC Symmetry trap column (180 μm i.d. ×2 cm packed with 5 μm C18 resin; Waters). Tryptic peptides were separated by reversed phase UPLC on a BEH C18 nanocapillary analytical column (75 μm i.d. ×25 cm, 1.7 μm particle size; Waters) using a 95 min nonlinear gradient formed by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 600 nL/min. The elution gradient was as follows: 6–9% B for 8 min, 9–14% B for 16 min, 14–30% B for 36 min, 30–40% B for 15 min, 40–95% for 10 min, and 95–96% for 5 min. The eluted peptides were analyzed using Q Exactive HF MS/ MS (ThermoFisher Scientific, Waltham, MA, USA). The MS full scans were performed in the ultrahigh-field Orbitrap mass analyzer in m/z ranges of 300–2,000 in positive ion mode with a resolution of 120,000 at m/z 200. The maximum injection time was 80 ms and the automatic gain control was set to 3×10^6. The MS/MS spectra were searched against the UniProt rat database (www.uniprot.org) for protein identification by use of Mascot 2.5.1 (Matrix Science, Boston, MA, USA). The false discovery rates for protein and peptide identifications were set at 1%.

Label-free relative quantification of proteins was achieved based on the MS data. The area under the curve of the peak of each peptide sequence is proportional to the abundance of the peptide. By use of the label-free quantification algorithm implemented in Mascot, the intensities of all representive peptides of a protein were integrated into a composite intensity to represent the relative abundance of the protein. Then, we divided

the composite intensity of each protein with that of adiponectin to represent the capacity of the protein binding to adiponectin.

Effect of differentially treated APN upon AMPK phosphorylation in HUVECs

The endothelial cell culture solution was mixed with apolipoprotein A1 (APOA1), apolipoprotein A4 (APOA4), apolipoprotein C1 (APOC1), or paraoxonase 1 (PON1), and incubated with recombinant APN (Abcam) at 37°C for 4 h. All ELISA kits were purchased from Sigma (St. Louis, MO, USA) or Abcam. The HUVECs were serum-starved for 3 h, and randomly treated with the following reagents (three wells per group): control group (normal culture medium), APN group (APN final concentration 10 μg/mL), APN+APOA1 group (respective concentrations of APN and APOA1 were 10 μg/mL and 20 μg/mL), APN+APOA4 group (respective concentrations of APN and APOA4 were 10 μg/mL and 5 μg/mL), APN+APOC1 group (respective concentrations of APN and APOC1 were 10 μg/mL and 10 μg/mL), APN+PON1 group (respective concentrations of APN and PON1 were 10 μg/mL and 2 μg/mL), APOA1 group (APOA1 concentration was 20 μg/mL), APOA4 group (APOA4 concentration was 5 μg/mL), APOC1 group (APOC1 concentration was 10 μg/mL), and PON1 group (PON1 concentration was 2 μg/mL). The concentrations of the above proteins were selected after literature review[6-9]. After 1 h treatment, the HUVECs in different groups were gathered and lysed. Proteins were extracted by centrifugation. Protein concentrations were determined by the BCA method. Protein concentrations were adjusted for consistency by adding loading buffer.

The AMPK and p-AMPK expression was determined by western blotting. The cultured cells were lysed with 1× cell lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin; Cell Signaling, Danvers, MA, USA) supplemented by a protease inhibitor cocktail (ThermoFisher Scientific). Nuclear and cytoplasmic proteins were isolated using a nuclear and cytoplasmic extraction kit (ThermoFisher Scientific). The protein samples were separated via SAS-PAGE electrophoresis, transferred to a polyvinylidene fluoride membrane, and blocked with 5% milk for 1 hour. The membrane was incubated overnight with primary antibodies for AMPKα (1:1,000) and p-AMPKα Thr172 (1:1,000) at 4°C. The membranes were then incubated with secondary horseradish peroxidase-conjugated secondary antibody (anti-rabbit antibody, 1/10,000; Cell Signaling) at room temperature for 2 hours. The western blotting bands were quantified by densitometry using ImageJ software (Image Lab, Bio-Rad).

Statistical analyses

Experimental data were expressed as $\overline{x} \pm$ s. Statistical analyses were performed via Prism 5.0 statistical software. The *t*-test was used for inter-group comparison, and *P* values <0.05 were considered statistically significant.

RESULTS

Effects of high-fat diet upon experimental animal plasma cholesterol and APN

After 8 weeks of feeding, the levels of plasma TC, TG, HDL-C, LDL-C, APN, and blood glucose in rats fed the high-fat diet were significantly greater than the levels in those fed the normal diet ($P < 0.05$, $P < 0.01$) (Figure 1). As expected, the body weight of the high-fat-diet group (352.9 ± 21.3) was significantly higher than that of the normally fed group (279.2 ± 19.1).

Comparison of levels of APN-binding proteins in normal plasma and high-fat plasma

Mass spectrometry demonstrated significantly increased binding of 18 proteins to APN in the high-fat plasma compared with the normal plasma (Table 1). Interestingly, among these APN binding proteins, four of them (including APOA1, APOA4, APOC1, and PON1) caught our special attention as they are critically involved in lipid metabolism. In the serum assays, the APOA1 levels were significantly increased in the high-fat plasma compared with the normal plasma content ($P < 0.05$). The levels of APOA4 and APOC1 were modestly increased, whereas PON1 was slightly decreased in the high-fat plasma compared with the control (not statistically significant, Figure 2).

AMPK phosphorylation in HUVECs undergoing different treatments

Many studies have confirmed that APN exerts a metabolic regulatory function primarily via AMPK phosphorylation[10]. Therefore, the biological activity of APN can be evaluated through the detection of AMPK phosphorylation. The AMPK phosphorylation levels in the exogenous APN treated group were significantly greater compared with the control group ($P < 0.05$ or $P < 0.01$, Figure 3). However, pre-incubation of APN with APOC1 (Figure 3b) or APOA1 (Figure 3c), but not APOA4 (Figure 3d) or PON1 (Figure 3a), significantly inhibited APN-induced AMPK phosphorylation compared with the regular APN group ($P < 0.01$ and $P < 0.05$). Taken together, these results demonstrated that binding of APN with APOA1 and APOC1 inhibit APN activity.

DISCUSSION

Adiponectin is well established for its favorable functions including insulin sensitization, myocardial protection, improvement of endothelial function, and anti-inflammation[1]. Hypoadiponectinemia is associated with endothelial dysfunction and atherosclerosis[11,12]. The mechanisms may include ameliorating lipid metabolism[13] and reducing lipid accumulation in macrophage foam cells[14]. In particular, a recent study by Kakino *et al.* shed light on the underlying mechanism with the finding that APN binds and inactivates atherogenic-LDL in humans[15]. However, recent studies have demonstrated that in the early stage of metabolic syndrome an increased APN level and

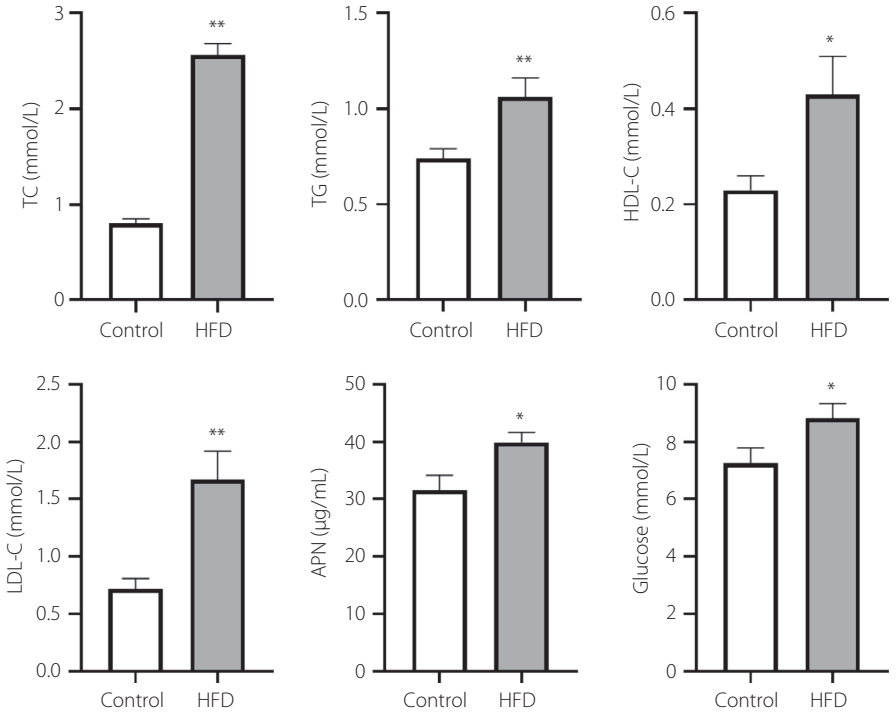

Figure 1 | Effects of a high-fat diet upon TC, TG, HDL-C, LDL-C, APN, and blood glucose levels in experimental rats. APN, adiponectin; HDL-C, high density lipoprotein cholesterol; HFD, high-fat-diet group; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride. *$P < 0.05$, **$P < 0.01$ vs control rats, $n = 10$. P values are generated from t-tests. The columns and error bars present the mean and SD.

impaired APN function coexist[16]. Given that research on the mechanisms responsible for this paradox is rare thus far, our study provides a novel pre-receptor explanation for the APN signaling resistance by the major components of HDL.

APN resistance occurs in insulin-sensitive skeletal muscle and liver tissue[17,18]. Additionally, APN resistance occurs during the early stages of type 2 diabetes[19]. The mechanisms of APN resistance are incompletely understood. Down-regulation of the APN receptor AdipoR1 expression in the musculature of patients with chronic heart failure is an important mechanism of APN resistance at the receptor level[20]. The increased plasma (Figure 1) APN is likely a compensatory mechanism for APN resistance in early-stage hyperlipidemia. Following this logic, our previous observation that plasma adiponectin increased soon after high-fat diet feeding[5] may imply that APN resistance responds rapidly to the increase in HDL induced by high-fat diet feeding. This is in concert with other findings that APN resistance occurs very rapidly after saturated fatty acid feeding independent of a change in AdipoR1 protein content[16]. Thus, we speculate that adiponectin resistance is likely to precede insulin resistance and to represent the triggering factor of the vicious circle formed by adiponectin resistance and insulin resistance in high-fat-diet induced metabolic disturbance.

Previous studies have demonstrated that the integrity of the vascular endothelium is the basis for the maintenance of normal blood flow, vascular permeability, and inflammatory response[21]. Hyperlipidemia increases superoxide production in vascular tissues, and inhibits the activity of eNOS of vascular tissue, causing endothelial cell damage and dysfunction[22]. APN protects the vascular endothelium via activating AMPK signaling, reducing superoxide generation, and regulating eNOS expression in endothelial cells[23]. Previously, we proved that early-stage hyperlipidemic rats exhibited significantly increased plasma APN[5], with levels reaching a peak at 8 weeks of high-fat diet feeding and decreasing abruptly afterwards, but APN-induced AMPK and eNOS phosphorylation in vascular endothelial cells did not concomitantly increase, suggesting an impairment in APN activity. Further studies have confirmed that a high-fat environment is detrimental to the protective effect of APN upon the vasculature, evidenced by reduced vascular endothelial AMPK and eNOS phosphorylation, and attenuated reduction of endothelial TNF-α and apoptosis, while the expression of APN receptors AdipoR1 and AdipoR2 on endothelial cells remained unchanged[5]. These studies suggest that substances in high-fat plasma inhibit APN activity and lead to APN function impairment at the pre-receptor level.

The present study demonstrated that 18 hyperlipidemic plasma molecules showed increased affinity to APN compared with that in normal plasma (Table 1). Among these proteins, we focused on the four proteins including APOA1, APOA4, APOC1, and PON1 that are actively involved in lipid metabolism. In addition, the APOA1 level in high-fat-diet group was

Table 1 | Mass spectrometry results

Protein	Diet group	$\bar{x} \pm s$	*P*
Apolipoprotein A4	HFD	365.152 ± 78.516	0.033
	ND	196.930 ± 22.129	
Apolipoprotein A1	HFD	141.131 ± 8.497	0.03
	ND	76.918 ± 29.847	
Serine protease inhibitor A3K	HFD	308.350 ± 115.361	0.01
	ND	78.057 ± 12.754	
Serotransferrin	HFD	188.299 ± 55.948	0.031
	ND	92.96 ± 31.555	
Apolipoprotein C1	HFD	25.361 ± 10.513	0.008
	ND	2.801 ± 0.116	
Hemopexin	HFD	51.106 ± 8.026	0.009
	ND	22.437 ± 7.519	
Protein Ighg3	HFD	3819.725 ± 1036.731	0.024
	ND	1933.442 ± 88.182	
Paraoxonase 1	HFD	16.806 ± 4.150	0.006
	ND	4.636 ± 1.568	
Plasminogen	HFD	6.910 ± 0.852	0.017
	ND	3.520 ± 0.950	
Isoform 2 of murinoglobulin-1	HFD	223.218 ± 69.743	0.044
	ND	121.883 ± 24.491	
Inter-alpha-trypsin inhibitor heavy chain H3	HFD	37.223 ± 9.039	0.011
	ND	12.854 ± 3.161	
Heparin cofactor 2	HFD	8.167 ± 3.719	0.028
	ND	2.804 ± 0.576	
Gelsolin	HFD	20.985 ± 0.479	0.026
	ND	11.988 ± 1.789	
Histidine-rich glycoprotein	HFD	11.189 ± 3.255	0.04
	ND	5.416 ± 1.754	
Inter-alpha trypsin inhibitor, heavy chain 1	HFD	18.232 ± 2.833	0.015
	ND	8.795 ± 0.656	
Protein Itih2	HFD	28.382 ± 2.97	0.012
	ND	13.166 ± 1.885	
Serum amyloid A protein	HFD	5.999 ± 0.912	0.001
	ND	1.169 ± 0.950	
Igh-6 protein	HFD	44.639 ± 5.393	0.013
	ND	22.955 ± 0.0209	

The values in $\bar{x} \pm s$ format are relative intensities which were intensity measurements of the corresponding plasma proteins divided by adiponectin intensity. *P*-values are from *t*-tests of comparison of the values between the high-fat-diet (HFD) and normal-diet (ND) groups. This table shows that the 18 proteins binding to APN significantly increased in the HFD group compared with the ND group analyzed by mass spectrometry ($P < 0.05$). $n = 4$.

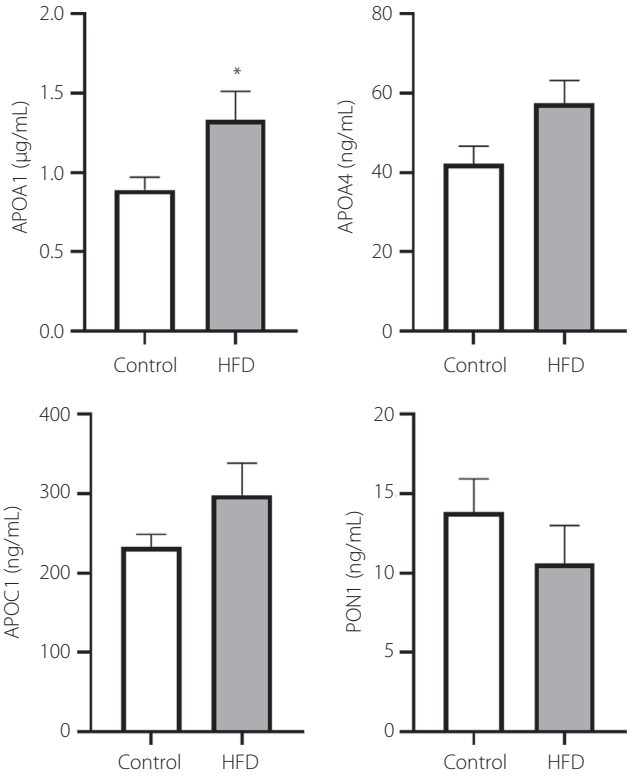

Figure 2 | Comparison of the levels of APN-binding vasoactive substances in the plasma of rats fed normal or high-fat diet. HFD, high-fat-diet group. *$P < 0.05$ vs control rats, $n = 6–8$. *P* values are generated from *t*-tests. The columns and error bars present the mean and SD.

significantly higher than that in the control group (Figure 2). It was further demonstrated for the first time that treatment with APOA1 and APOC1 can significantly inhibit the biological activity of APN in terms of AMPK phosphorylation *in vitro* (Figure 3). Collectively, our results implied that APOA1 and APOC1 may play important roles in the inhibition of APN activity in early-stage hyperlipidemia. This pre-receptor mechanism of APN dysfunction may provide a novel clue for the development of intervention, targeting to restore the impaired APN signaling in hyperlipidemia and thus rescue cardiovascular dysfunctions.

APOA1 and APOC1 are the major components of plasma HDL. Studies have suggested a positive correlation between plasma HDL and plasma APN in patients with type 2 diabetes[24]. Traditionally, HDL has been regarded as a vascular protective factor, performing various anti-atherosclerotic and anti-inflammatory functions. However, the most recent study demonstrated that HDL increases the intracellular ceramide concentration[25]. Intracellular ceramide accumulation affects the phosphorylation of protein kinase B (Akt) and Akt substrate 160[26], thereby impairing cellular glucose transportation and disrupting energy metabolism. The increase of plasma APOC1 induces aortic smooth muscle cell apoptosis by sphingomyelinase recruitment[27], suggesting that HDL and its components may play different roles during the development of cardiovascular diseases and complications. Our current study demonstrating APOA1/APOC1 and APN binding and APN functional inhibition provided a novel mechanism contributing to the diverse/controversial (protective and harmful) functions of high-density lipoprotein.

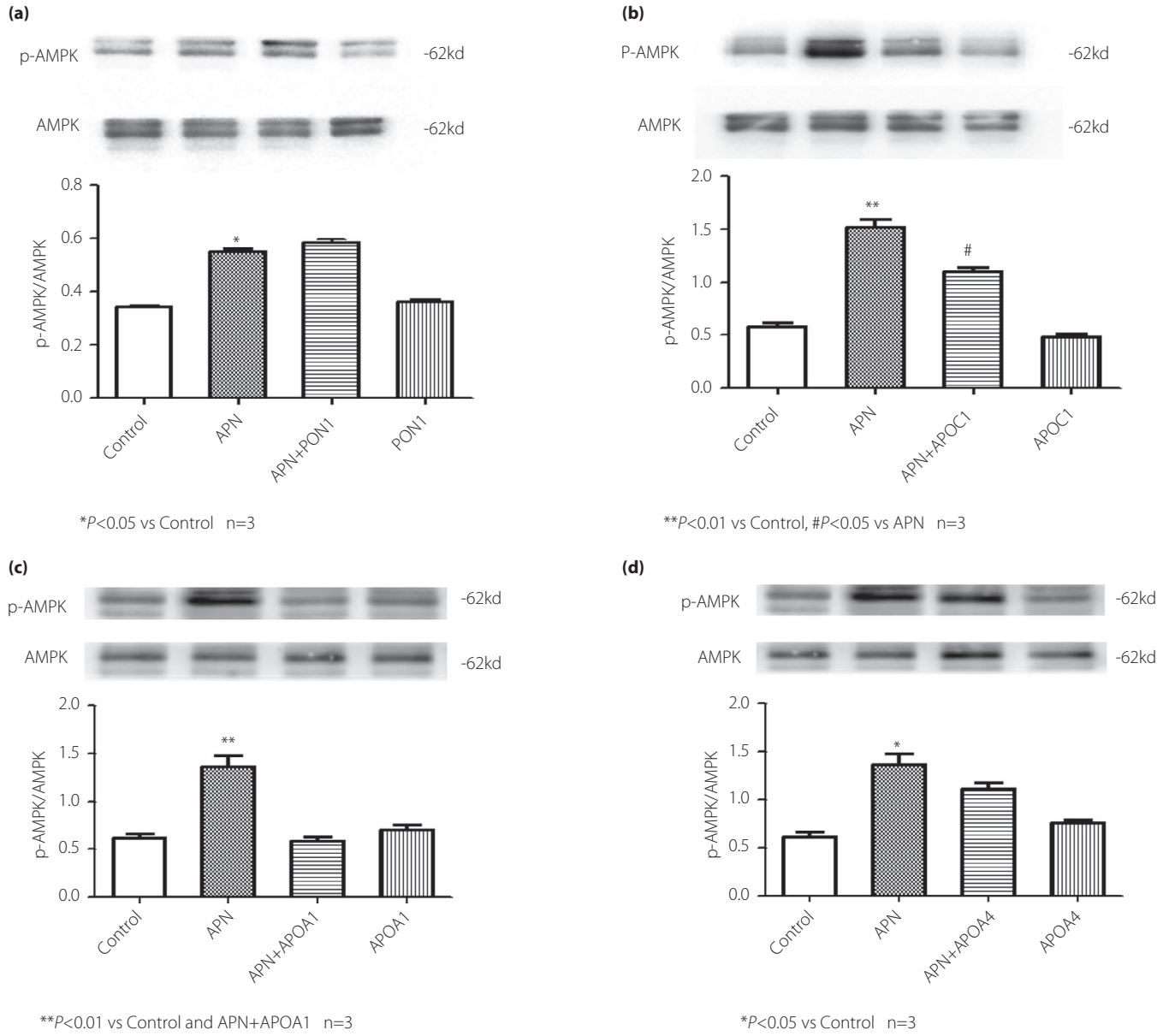

Figure 3 | Comparison of p-AMPK/AMPK ratio in human umbilical vein endothelial cells pre-treated with adiponectin along with PON1 (a), APOC1 (b), APOA1 (c), or APOA4 (d). Both p-AMPK and AMPK were determined by western blot. p-AMPK, phosphorylated AMPK. *P* values are generated from *t*-tests. The columns and error bars present the mean and SD.

The major limitation of this study is that we only further investigated 4 of the 18 molecules with increased binding to APN in hyperlipidemic plasma regarding whether they inhibited AMPK phosphorylation. We selected these four molecules because they have the biggest potentials to inhibit adiponectin functions based on our research[4,5] and a literature review[28]. However, inclusion of all 18 molecules may generate a more comprehensive conclusion. Another limitation may lie in the potential difference in the relationship of lipid metabolism and atherosclerosis between rodents and human, given that rodents are naturally deficient in cholesteryl ester transfer protein (CETP) activity[29]. Rats were used in the *in vivo* experiments, so the related findings warrant further testing in human, although the expression of a human CETP gene in mice was proved to play a protective role as in humans[30].

In summary, our results demonstrate for the first time that increased levels of APOA1 and APOC1 in high-fat plasma binds APN, inhibiting its biological activity. This may represent a novel pre-receptor mechanism of APN function impairment besides the well-known hypoadiponectinemia and post-receptor adiponectin resistance, which mechanisms may jointly lead to endothelial dysfunction and cardiovascular complications

during early stage hyperlipidemia. These results help to develop new strategies for treating and preventing resultant metabolic syndrome and cardiovascular diseases.

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DISCLOSURE

The authors declare no conflicts of interest.
Approval of the research protocol: N/A.
Informed consent: N/A.
Approval date of registry and the registration no. of the study/-trial: N/A.
Animal studies: All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals. This work was approved by the Air Force Medical University Committee on Animal Care on July 5 2015. The approval number is 20150302.

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