Homocysteine promotes migration of adventitial fibroblasts via Angiotensin II type 1 receptor activation to aggravate atherosclerosis

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Research article

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Abstract

Background

Hyperhomocysteinemia (HHcy) is an independent risk factor for atherosclerosis. However, the mechanisms of HHcy-induced arteriosclerosis are largely unknown. Objective: To clarify the effect of Hcy on adventitial fibroblasts (AFs) and its relation with angiotensin II type 1 receptor (AT1R).

Method:

The apolipoprotein E gene-deficient mice (ApoE^{-/-}) were used as the murine model for atherosclerosis. HHcy was induced and treated by feeding them 1.5% methionine and telmisartan (gavage 10 mg/kg/d) for 12 weeks, respectively. The AFs and HEK293A cells transfected with the AT1R plasmid were used to investigate the interaction between Hcy and AT1R. All data were expressed as mean $\pm$ SEM. The data were analyzed by one-way ANOVA or repeated measurement ANOVA.

Results:

HHcy aggravated the plaque area of the aortic root and increased the expression of IL-6, MCP-1, and the macrophage marker Mac3 in the plaque and adventitia of the aorta, whereas telmisartan improved this effect. HHcy induced the occurrence of the AFs marker protein ER-TR7 in the plaque and the entire layer of the aorta, whereas telmisartan also improved this effect, indicating that HHcy induced AFs migration and that AT1R mediated this process. Hcy increased the production of AFs H₂O₂, ROS, and IL-6 of AFs, indicating that Hcy activated the oxidative stress and inflammatory reactions, which may induce cell migration. The subsequent scratch and transwell experiments confirmed that Hcy induced the AFs migration and that telmisartan inhibited this effect. Hcy increased the expression of AFs AT1R and the phosphorylation levels of PKC and ERK1/2 in the AF and HEK293A cells transfected with the AT1R plasmid, whereas telmisartan inhibited this effect, indicating that Hcy activated AT1R intracellular signaling pathway.

Conclusion:

Hcy promoted adventitial inflammation, induced AFs migration, and aggravated atherosclerosis by activating AT1R.

Background

Hcy is a nonessential sulfur-containing amino acid derived from the essential amino acid methionine and actively participates in various biochemical reactions.¹ HHcy (circulating Hcy $\geq 15 \mu\text{m}$) is an independent risk factor for many cardiovascular diseases, such as atherosclerosis, coronary heart disease, and abdominal aortic aneurysm. Although there is evidence that HHcy can cause vasodilation, damage endothelial cells, promote intravascular proliferation, promote outer membrane activation, and

disrupt hemostasis/coagulation,²⁻⁵ the mechanism by which Hcy aggravates atherosclerosis remains elusive.

AT1R is a key player in the renin-angiotensin system. AT1R activation leads to cardiac remodeling, ventricular hypertrophy, intimal formation, smooth muscle cell proliferation, and migration.^{6,7} Genetic deletion of AT1R effectively prevents pathological vascular injuries in various animal models, such as models hypertension and atherosclerosis.⁸ Although recent studies indicate Hcy directly interacts and activates AT1R to aggravate vascular injury in abdominal aortic aneurysm (AAA) mouse model¹ and Hcy accelerates collagen accumulation in the adventitia of balloon-injured rat carotid arteries via AT1R expression,⁹ little is known about the association between Hcy and AT1R in atherosclerosis.

Migration and proliferation responses of cells in the vascular wall and deposition of extracellular matrix (ECM) play a key role in restenosis and atherosclerosis.¹⁰ Although medial smooth muscle cells have been regarded as the main source of cells,^{11,12} there is now increasing evidence that the adventitial layer can also be a significant contributor to the arterial remodelling process through increased angiogenesis.¹³ Findings by Richard C.M. et al.,¹⁰ provide direct evidence that adventitial fibroblasts migration contribute to neointima formation after balloon injury. Hcy induced AT1R expression that activates various signaling molecules extracellular signal-regulated protein kinases 1 and 2 (ERK-1/2) and protein kinase C (PKC). These signaling molecules activation can promote cell migration and invasion.¹⁴⁻¹⁶ However, the precise role of Hcy in the migration and invasion of adventitia fibroblasts is unclear in the process of atherosclerosis formation.

Therefore, we investigated whether HHcy stimulates AFs migration and invasion in mice via AT1R activation to accelerate the formation atherosclerosis.

Methods

Materials

L-homocysteine (69453), telmisartan (T8949), was purchased from Sigma-Aldrich. Disposable consumables such as cell culture bottle and 6-well plates were purchased from Guangzhou Jet Bio-Filtration Co., Ltd.

Animals and Treatments

A total of 21 ApoE^{-/-} mice (male, 8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Beijing, China (Ltd. Number: SCXK2016-0006). The mice were randomly divided into three groups: a control group with a standard mouse diet (n = 7), an HHcy group fed with standard mouse diet plus 1.5% methionine (n = 7),^{17, 18} and a telmisartan gavage group (HHcy+Telmi) treated with telmisartan (10mg/kg/day).¹⁹ Ten Sprague Dawley (SD) rats (male, weighing 150–200 grams) were purchased from Inner Mongolia Medical University Laboratory (Hohhot, China). All animal experiments

were approved by the Institutional Animal Care and Use Committee (No: YKD2015110) of Inner Mongolia Medical University, Hohhot, China and was conducted in accordance with the Guide for the Care and Use of laboratory animals from the National Institutes of Health (Bethesda, MD, USA).

Tail-cuff measurement of systolic blood pressure

Systolic blood pressure (SBP) was recorded by a computerized non-invasive tail-cuff system (Cat. NO. BP-300A, Chengdu Techman Software CO., LTD). Measurements were performed in quiet environment to avoid causing mice anxiety. The mice underwent 7 consecutive days of training sessions to become accustomed to the tail-cuff procedure. Blood pressure of mice underwent 12 consecutive weeks was measured from 1 to 6 PM on weekends every week. Three measurements were weekly performed on each mouse, so that the average of total of 3 measurements was represented as the SBP of each mouse.¹

Measurement of Serum Homocysteine Level

The levels of TG, TC, HDL, and LDL were assessed with kits from Beijing Sino-UK Institute of Biological Technology. Hcy concentration was measured using enzyme-linked immunosorbent assay (HY-N0080) in Beijing Sino-UK Institute of Biological Technology.

Histology and Immunostaining

The heart and approximately 2 mm of proximal aortas were fresh frozen in OCT compound.²⁰ Tissue samples were cut into 7µm thin slices. Frozen tissue sections were stained with Oil Red O, H&E, Masson, and immunostaining.²¹ A Masson's trichrome staining kit was used to detect collagen deposition in the atherosclerotic plaque. Analyzed with Image-Pro Plus 6.0 or ImageJ software.²⁰

Cryosections were fixed in 4% paraformaldehyde and subsequently treated with H₂O₂ and after the primary antibody was blocked in 5% goat serum (1:100) at 4°C overnight. The tissue sections were washed in PBS then incubated with secondary antibody (1:100). They were incubated in diaminobenzidine until a change in color was observed under the microscope and then the nucleus was stained with hematoxylin.²² Immunofluorescence staining procedure was similar to immunostaining.

Cell Culture

Primary Rat AFs

Cells were prepared according to common methods²³ and with some modifications. SD rat were euthanized with sodium pentobarbital at 120 mg/kg. Thoracic aortas were removed and cleaned under sterile conditions. The adventitia was stripped from the aorta and cut into pieces about 2–3 mm³ in size. Then, the tissue pieces were attached to the 6-well plates and immersed in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 30% fetal bovine serum (FBS, Gibco) and maintained at 37°C, 5% CO₂. Cells were collected, and when the cells grew to about 70%–80% fusion, the tissue pieces could be cultured again. AFs were verified by positive immunofluorescent staining of vimentin and ER-TR7. The

AFs after passage were cultured in DMEM supplemented with 10% FBS at 37°C, 5% CO₂. All AFs used in this study were from early passage with a maximum of four passages.

The human embryonic kidney cell line HEK293A was a generous gift from our laboratory and cultured in DMEM (high glucose) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. The cells were confirmed of no mycoplasma contamination before application.

Cell Proliferation Assay

The proliferation of cells was examined with Cell Counting Kit-8 (Cat. NO. K1018, ApexBio Technology LLC). Briefly, cells were seeded in 96-well plates with 5×10^3 cells/well, cultured for 24 hours, and subjected to the indicated treatment.²⁴ The absorbance at 450 nm was measured using an automatic microplate reader (n = 3).

Wound-Healing Assay

AFs (5×10^5 cells/well) were inoculated into 6-well plates. After the cells completely covered the bottom wall of the 6-well plate, three vertical lines were drawn in the 6-well plate with a sterile 200- μ l pipette tip, and the floating cells were removed by PBS washing.²⁵ DMEM 1000 μ l containing 1% FBS and Hcy 200 μ mol/L was added into the 6-well plate. The cells were cultured in an incubator at 37°C and 5% CO₂ for 48 hours. At the same location, the images of 0 and 48 hours were taken to observe the cell migration. Cell migration analysis was processed by Image-Pro Plus 6.0 software²⁵ (n = 3).

Transwell Matrigel Invasion Assays

Transwell Matrigel invasion assays were performed using a transwell membrane (8- μ m pore size, 6.5-mm diameter; Cat. NO. 35488, Corning Incorporated) in a 24-well plate. The Hcy-treated cells were digested with trypsin (0.25%) and then centrifuged and the supernatant was discarded. The cell suspension was prepared with DMEM without serum. The cells (1×10^5 cells/well) were loaded to the upper side of the chamber (500 μ l/well), and the lower chambers contained 750 μ l/ of DMEM with 10% FBS as chemoattractant. The cells were cultured in the cell incubator for 6 hours. The filter inserts were then removed from the wells. Cells on the upper surface of the filter were removed using cotton swabs and the membranes were fixed and stained with crystal violet reagent.^{21,26} Invasion was quantified by counting 5 random fields under a light microscope (Leica, Germany) at x40 magnification.

Detection of Intracellular ROS

Intracellular ROS generation was monitored by using Reactive Oxygen Species Assay Kit (WLA070a, Wanleibio). Cells (1×10^6 cells/well) were seeded in 6-well plates. Cells were treated with Hcy for 48 hours and stained with 20 μ mol/L DCF-DA in culture medium for 30 minutes in the dark. The cells were then

harvested and washed in PBS three times and oxidation-induced DCF fluorescence was assayed by fluorescence microscopy.²⁷

Assay of Hydrogen Peroxide Level

Hydrogen peroxide level was assayed using a Hydrogen Peroxide Assay Kit (Cat. NO. A064-1-1, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

Western Blot Analysis

Protein extracts (40 µg) were resolved by 10% SDS-PAGE for western blot analysis. The blots were subsequently incubated with primary antibodies (AT1R, 1:300, Abcam, ab124734; p-PKC, 1:1000, Cell Signaling Technology, 9371s; t-PKC, 1:1000, Wanleibio, WL02234; p-ERK1/2, 1:1000, Cell Signaling Technology, 9101s; t-ERK1/2, 1:1000, Cell Signaling Technology, 9102s). The corresponding secondary antibody was added for incubation at room temperature. The protein expressions in different samples were detected using the enhanced chemiluminescence method.

Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM), except Table 1 and table 2 (mean ± standard deviation). All experiments were repeated at least three times. The data were analyzed by GraphPad Prism 5.0 (GraphPad Software, San Diego, California, USA) and SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed by one-way ANOVA or repeated measurement ANOVA, without assuming sphericity. When multiple measurements were made in the same cell, Greenhouse–Geisser correction was performed. The data that failed to pass the normality test were analyzed by nonparametric test. The difference was significant ($P < 0.05$).

Results

Vital parameters

HHcy was induced by feeding ApoE^{-/-} mice a high-methionine diet for 12 weeks. The results showed that serum Hcy concentrations in the HHcy group were significantly higher (35.89 ± 3.26 µmol/L) than the control group (23.85 ± 2.09 µmol/L), ($*P < 0.05$). Therefore, the high-methionine diet significantly increased serum concentrations of Hcy, suggesting that the diet-induced HHcy model was successful (Table 1). Total cholesterol (TC), and high-density lipoprotein (HDL) in HHcy group were significantly difference except triglyceride (TG). TC level in HHcy group was higher than that in control group ($P < 0.05$). TC decreased significantly after telmisartan treatment ($P < 0.05$), Low-density lipoprotein (LDL) level in HHcy group was higher than that in control group, but there was no statistical significance. After telmisartan treatment, LDL decreased significantly ($P < 0.05$), as shown in Table 1.

Telmisartan treatment decreased systolic blood pressure. In HHcy+Telmi group, the systolic blood pressure of mice after treatment was lower than that before treatment ($P < 0.01$); however, there was no

statistical difference in three groups, showing no significant changes in body weight (Table 2A and 2B).

Comparison of atherosclerotic plaque in aortic root of three groups of mice

A large amount of lipid deposition was found in the aortic root of mice in the HHcy group. The area of atherosclerotic plaque accounted for about (23.1%) of the aortic root, which was larger than that in the control group (6.3%), as shown by the Oil Red O staining. The plaque area in the HHcy+Telmi group (9.1%) was significantly lower than in the HHcy group (Fig. 1).

Comparison of the plaque instability in three groups of mice

Hematoxylin and eosin staining showed higher number of foam cells and a larger acellular necrotic core area in the HHcy group, which was improved in the HHcy+Telmi group (Fig. 2a). Moreover, Masson staining (Figs. 2b and 2c) showed that total collagen content, expressed as a percentage of the section area, was significantly reduced in HHcy group compared with that in control group ($P < 0.001$), while it was significantly increased in the HHcy+Telmi group.

Comparison of inflammation of atherosclerotic plaque in three groups of mice

The number of inflammatory cells such as MCP-1, IL-6, and Mac3 in aortic root plaques in HHcy group was significantly higher than that in control group ($P < 0.05$), while it was reduced in the HHcy+Telmi group (Figs. 3a and 3b).

Comparison of aortic inflammation in three groups of mice

Results showed that inflammatory cells MCP-1, IL-6, and Mac3 were preferentially produced in the adventitial layer. Quantitative analysis of aortic adventitia revealed higher expression of MCP1, IL-6, and Mac3 in the HHcy group than in control group. Furthermore, the AT1R blocker telmisartan markedly attenuated MCP1, IL-6, and Mac3 expression (Fig. 4), which is consistent with observations in a previous study.²

Expression of AFs marker protein ER-TR7 in aorta of three groups of mice

Immunofluorescence was used to detect AFs marker ER-TR7 (Fig. 5). In the control group, staining showed ER-TR7 expression in aortic adventitia. Moreover, deposition of ER-TR7 increased in the whole aortic wall and plaque in the HHcy group. This phenomenon was reduced by telmisartan, as shown in the HHcy+Telmi group.

Effect of Hcy on oxidative stress and inflammatory factors in AFs.

Our results showed that Hcy of 200 $\mu\text{mol/L}$ greatly enhanced hydrogen peroxide (H_2O_2) secretion (Fig. 6a) and increased intracellular ROS at 48 hours after stimulation (Fig. 6b). Hcy induced IL-6 expression in a time-dependent manner, and the most effective Hcy time was 36 hours as indicated by using western

blot (Figs. 6c and 6d). These results demonstrated that Hcy could activate AFs oxidative stress and inflammatory response.

Effect of Hcy on the expression of MMP-9 in AFs

Hcy induced MMP-9 expression in a time-dependent manner, and the most effective Hcy time was 36 hours as indicated by using western blot (Fig. 7). Matrix remodeling is a critical step to initiate and support AFs motility.

Effect of Hcy on proliferation, migration and invasion of AFs

AFs were treated with increasing concentrations of Hcy (0, 50, 100, 200, and 400 $\mu\text{mol/L}$) for 48 hours. Results showed that cell viability was increased significantly in a dose-dependent manner, indicating that Hcy promoted cell proliferation (Fig. 8a). Cells were treated with 200 $\mu\text{mol/L}$ Hcy for 48 hours. The wound areas of the cells were observed via scratch assay under a microscope at 0 hour and 48 hours. Results showed that Hcy significantly increased cell migration (Figs. 8b and 8c). Cell invasion was detected by transwell Matrigel invasion assays. In AFs stimulated by 200 $\mu\text{mol/L}$ Hcy for 48 hours, cell invasion was significantly increased (Figs. 8d and 8e). After telmisartan (10 $\mu\text{mol/L}$) pretreatment for 1 hour, this phenomenon was inhibited.

Effect of Hcy on expression of AT1R in AFs

Hcy induced AT1R expression in a time-dependent manner as indicated by using western blot (Fig. 9). There was a significant increase in AT1R expression at 36 hours (Figs. 9a and 9c). Moreover, Hcy of 50 to 300 $\mu\text{mol/L}$ significantly facilitated AT1R expression, with a peak at 200 $\mu\text{mol/L}$ (Figs. 9b and 9d). This phenomenon was further verified by immunofluorescence staining of AT1R protein (Fig. 9e).

Effect of Hcy on AT1R signaling

We assessed G protein-dependent signaling PKC and ERK1/2 phosphorylation in AFs. The phosphorylation of ERK1/2 and PKC was increased after stimulated with Hcy (200 $\mu\text{mol/L}$), which was decreased by telmisartan pretreatment (10 $\mu\text{mol/L}$) (Figs. 10a to 10c).

To further verify the results, we constructed EGFP-AT1R fusion protein particles and transfected HEK293A cells to assess the effect of Hcy on PKC and ERK1/2 signaling pathways. We used agarose gel electrophoresis to detect the extracted plasmids and transfected into HEK293A cells (Figs. 11a and 11b). Western blot indicated AT1R levels in HEK293A cells (Figs. 11c and 11d). The results confirmed the successful construction of the plasmids by GeneChem Co. Ltd. (Shanghai, China) (Fig. 11a). Fluorescence microscopy showed that the green fluorescence was much brighter in the HEK293A cells transfected with AT1R compared with the control group (Fig. 11b). Western blot indicated higher AT1R expression in plasmids transfected into HEK293A cells than in the control group (Figs. 11c and 11d).

These results indicated that the plasmid was successfully constructed and transfected into HEK293A cells.

In HEK293A cells transfected with AT1R, Hcy at 200 $\mu\text{mol/L}$ resulted in delayed but sustained PKC and ERK1/2 phosphorylation, which was decreased by telmisartan pretreatment (10 $\mu\text{mol/L}$) (Figs. 12a to 12c). These results suggested that Hcy could activate the downstream PKC and ERK1/2 signaling pathway downstream of AT1R.

Discussion

It is well known that Hcy can cause vascular injury, atherosclerosis, and AFs are involved in this process. Siow RC et al., proved that AFs can migrate from the outer membrane to the neointima after balloon injury in rats.¹⁰ The outer membrane plays a pathogenic role by producing large amounts of NADPH oxidase-derived ROS and recruiting inflammatory cells.²⁸ Hcy aggravated aortic adventitial inflammation, increased the incidence of AAA in ApoE^{-/-} mice caused by infused angiotensin II. The mechanism of this adverse event was that homocysteine activates NADPH oxidase 4 in adventitial fibroblasts.² Furthermore, HHcy exacerbated vessel remodeling following balloon injury in rats by increasing collagen deposition in adventitia.²⁹ However, valsartan, the AT1R antagonist could inhibit this phenomenon, implying the involvement of AT1R in the pathogenic effects of Hcy on adventitial remodelling.⁹ Genetic deletion of AT1a receptor effectively prevents pathological vascular injuries in a variety of animal models, including models of atherosclerosis and AAA.⁸ Recent data have demonstrated that Hcy directly interacted and activated with AT1R to aggravate vascular injury in AAA mouse models.¹ However, the underlying mechanisms of HHcy in promoting vascular inflammation and atherosclerosis remains unknown. Here, we demonstrated that HHcy induced AFs to participate in the formation of atherosclerosis, and the related mechanism may be related to AT1R (Table 3).

A plethora of evidence details the crucial role that dyslipidemia play in vascular pathology associated with HHcy.³⁰ Vascular mitochondrial oxidative stress increases under hyperlipidemic conditions, resulting in enhanced atherosclerotic plaque inflammation, necrotic core and fibrous cap remodeling, and susceptibility to rupture.²⁰ In this study, ApoE^{-/-} mice receiving methionine diet displayed high levels of serum Hcy, TC (Table 1), and typical atherosclerotic plaques, thereby causing accumulated lipid, increased necrotic core,¹⁷ decreased collagen fiber content, and infiltrated inflammation cells in the aortic root. After telmisartan treatment, these effects of HHcy were inhibited³¹ (Figs. 1-3). Consistent with the study by Schlimmer, our data revealed that telmisartan can effectively reduce oxidative stress in aortic tissue and subsequent peroxidation of membrane lipids, thereby preventing atherosclerosis formation.³² Based on these results, we speculated that Hcy might promote atherosclerosis formation via AT1R activation. Furthermore, lipid loading increased adventitial metalloproteinase-9 levels to promote cell migration.³³ Intriguingly, we found that HHcy induced AFs migration and invasion in vitro and in vivo. Telmisartan also inhibited the migration and invasion of fibroblasts induced by HHcy (Figs. 5 and 8). Consistent with Dan Yao,⁹ Hcy promoted AT1R expression in AFs in a time- and concentration-dependent

manner (Fig. 9). Hcy increased ROS production in AFs, increased H₂O₂ in cell supernatant, and promoted IL-6 and MMP9 expression in AFs (Figs. 6-7). ROS produced by NADPH oxidase is an important mediator in signal transduction.³⁴ Cell migration in its essence is an invasive process that requires degradation of ECM through activation of matrix metalloproteinases (MMPs).³⁴ Compelling evidence suggests that AT1R leads to ROS production.² Moreover, ROS can directly or indirectly activate MMPs, hence inducing cell migration. These results suggest that Hcy may promote AFs migration and invasion by enhancing AT1R expression and then accelerate atherosclerosis formation. The development of atherosclerosis depends largely on local inflammation. A large number of macrophages in atherosclerotic lesions is an indicator of a more unstable and rupture-prone phenotype.^{35,36} In fact, these cells are aggregated in plaques and swallow lipids, become foam cells, and release cytokines and growth factors, as well as MMPs and ROS that degrade the structures of plaques to provide conditions for cell migration.³⁷ Our study also showed that HHcy induced the infiltration of inflammatory cells in aortic root plaque and aortic adventitia inflammation in mice, which was consistent with Lili et al.² (Figs. 3-4). The results of mice aortic immunohistochemistry showed that infiltrations of MCP-1, IL-6, and Mac3 were mainly observed in the tunica adventitia of mice aorta. MCP-1 is a potent chemokine that stimulates cells migration.³⁸ There is increasing evidence suggesting that Hcy has a possible role in triggering arterial inflammation.^{2,39,40} ERK-1/2 activation has strongly been shown to be associated with inflammation^{41,42} and atherosclerosis.⁴³ A large number of studies have shown that ERK and PKC activation can promote cell migration and invasion.^{44,45} To further explore relevant mechanisms, AFs and HEK293A cells transfected with human AT1R were stimulated by Hcy, with or without telmisartan pretreatment for 1 hour, and the expression level of ERK-1/2 and PKC signaling pathway in AT1R was detected. Our results demonstrated that Hcy induced a robust and significant increase of ERK-1/2 and PKC phosphorylation, which was markedly attenuated by telmisartan (Figs. 11-12). These results clearly suggest the involvement of AT1R in mediating ERK-1/2 and PKC phosphorylation in Hcy.

Of note, we found that telmisartan can reduce Hcy-induced atherosclerotic plaque in vivo. Besides, it was shown that the concentration of Hcy was significantly decreased in the HHcy + Telmi group compared with that in the HHcy group in Table 1, and the systolic blood pressure of mice after treatment was lower than that before treatment in HHcy+Telmi group. Therefore, telmisartan might exert protective effects by decreased Hcy instead of AT1R inhibition or its antihypertensive effect (Table 2B). However, we did find that Hcy could up regulate the expression of AT1R in AFs and induce the migration and invasion of AFs. Telmisartan pretreatment could reduce this phenomenon. In this study, we did not further knockdown AT1R in AFs and evaluate its migration. However, previous animal experiments have confirmed that the atherosclerotic area of ApoE -/- / AT1a -/- mice is smaller than that of ApoE -/- .⁴⁶ So, Hcy at least partially activates AT1R, induces AFs migration and invasion, and aggravates the formation of atherosclerosis.

In summary, our findings suggest that Hcy induces AT1R and ERK-1/2 and PKC activation. Activated ERK-1/2 and PKC further contribute to AFs proliferation, migration, and invasion and participate in the process of atherosclerosis by modulating MMP-9.

Conclusion

In conclusion, our experiments confirm that HHcy aggravates atherosclerosis formation, at least in part, by activating AFs through AT1R, causing AFs proliferation, migration, and invasion. These findings suggest that strategies designed to block AT1R may reduce HHcy-related adventitial migration and invasion and thus reduce HHcy-related vascular diseases.

Abbreviations

Hcy	homocysteine
HHcy	hyperhomocysteinemia
AFs	adventitia fibroblasts
AT1R	angiotensin II type 1 receptor
Apoe ^{−/−}	apolipoprotein E gene-deficient mice
IL-6	interleukin-6
MCP-1	monocyte chemoattractant protein-1
Mac3	macrophages
H ₂ O ₂	hydrogen peroxide
ROS	reactive Oxygen Species
MMP-9	matrix metalloproteinase
PKC	protein kinase c
ERK1/2	extracellular signal-regulated protein kinases 1 and 2
ECM	extracellular matrix
Telmi	telmisartan
SD	sprague dawley
SBP	systolic blood pressure
TG	triglyceride
TC	total cholesterol
LDL	low-density lipoprotein
HDL	high-density lipoprotein
SEM	standard error of the mean

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (No: YKD2015110) of Inner Mongolia Medical University, Hohhot, China and was conducted in accordance with the Guide for the Care and Use of laboratory animals from the National Institutes of Health (Bethesda, MD, USA).

Consent for publication

Not applicable

Availability of data and material

The datasets used and/ or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no competing interests exist.

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Authors' contributions

ZBZ designed the experiments, carried out the experiments and wrote the manuscript; XY H and TXL wrote the manuscript; JQG designed the experiments revised the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1. Hcy and lipid values of three groups

	Hcy	TG	TC	LDL	HDL
	($\mu\text{mol/L}$)	($\mu\text{mol/L}$)	($\mu\text{mol/L}$)	($\mu\text{mol/L}$)	($\mu\text{mol/L}$)
Control	23.85 $\pm$ 2.09	1.07 $\pm$ 0.53	10.26 $\pm$ 1.47	3.03 $\pm$ 0.57	1.64 $\pm$ 0.27
HHcy	35.89 $\pm$ 3.26*	1.21 $\pm$ 0.36	12.72 $\pm$ 0.55*	3.61 $\pm$ 0.72	1.27 $\pm$ 0.21*
HHcy+Telmi	17.32 $\pm$ 3.94 [#]	0.88 $\pm$ 0.28	8.76 $\pm$ 1.59 [#]	2.68 $\pm$ 0.31 [#]	1.95 $\pm$ 0.11 [#]

Hcy and lipid levels were measured in five to seven mice per group. Data represent mean $\pm$ SEM

*P < 0.05 compared to control group

#P <0.05 compared to HHcy group

Control, standard mouse diet group; HHcy, 1.5% methionine group; HHcy+Telmi, HHcy + telmisartan treatment group; HHcy, homocysteinemia; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein

Table 2A. Weight of three groups

	Control	HHcy	HHcy+Telmi	P ₁
Weight before (g)	22.74	22.79	23.19	0.32
	±1.46	±0.92	±0.81	
Weight after (g)	26.73	28.66	25.89	0.08
	±0.91	±1.29	±1.29	

Table 2B. Systolic blood pressure of three groups

	Control	HHcy	HHcy+Telmi	P ₁
SBP before (mmHg)	119	125	121	0.31
	±4.97	±10.29	±9.01	
SBP after (mmHg)	120	121	113**	0.16
	±4.41	±6.08	±7.05	
P ₂	0.23	0.15	0.00	

Weight and SBP were measured in five to seven mice per group. Data represent mean ± SD.

Control, standard mouse diet group; HHcy, 1.5% methionine group; HHcy+Telmi, HHcy + telmisartan treatment group; SBP, systolic blood pressure.

Weight before: the weight of mice without 1.5% methionine diet and telmisartan gavage. Weight after: the weight of mice underwent 12 consecutive weeks after receiving 1.5% methionine diet and telmisartan gavage.

SBP before: the systolic blood pressure of mice without 1.5% methionine diet and telmisartan gavage. SBP after: the systolic blood pressure of mice underwent 12 consecutive weeks after receiving 1.5% methionine diet and telmisartan gavage.

P₁: Comparison among the three groups. P₂: Before and after treatment in each group.

**P < 0.01 compared to SBP before in HHcy+Telmi group.

Table 3. Comparison of different experimental models in References

	Li T et al., 2018	Liu Z et al., 2012	Yao Det al., 2014	Fukuda D et al., 2010	manuscript
Animal models	HHcy model AAA model mice	HHcy model AAA model mice	HHcy model Balloon-Injured Rat Carotid Arteries	a western-type diet to induce atherosclerosis model	HHcy model atherosclerosis model
Cell models	Hcy stimulated HEK293A cells transfected with the AT1R plasmid to determine AT1R signaling.	Hcy stimulated rat AFs to explore the mechanism of aortic adventitia inflammation.	Hcy stimulated rat AFs to explore the expression of collagen type 1 and AT1R.		Hcy stimulated rat AFs or HEK293A cells transfected with the AT1R plasmid to explore the mechanism of AFs migration
Mechanisms	Hcy directly activates AT1R signalling to aggravate vascular injury.	Hcy may aggravate AAA formation at least partially via activating AFs NADPH oxidase 4.	Hcy regulates collagen type 1 expression of in AFs to exacerbate adventitial remodeling via AT1R.	Telmisartan has protective effects beyond AT1R blockade, which is most likely PPARγ activation	Hcy promotes adventitial inflammation, induces AFs migration, and aggravates atherosclerosis via AT1R signaling.
Potential applications	Strategies aimed at blocking the AT1R may mitigate HHcy- associated aneurysmal vascular injuries.	Hcy directly activates AT1R signalling to aggravate vascular injury.	Blocking AT1R may have potential promising benefit of AT1R for patients with HHcy.	Multiple effects of telmisartan (i.e. AT1R blocking and PPARγ agonist) are useful for management of patients.	Strategies designed to block AT1R may reduce HHcy-related atherosclerosis diseases. It can also delay the progress of atherosclerosis by reducing vascular inflammation.

HHcy, homocysteinemia; Hcy, homocysteine

Figures

Figure 1

Hcy accelerates atherosclerosis formation (a) Representative Oil Red O staining of the thoracic aorta root sections. (b) Quantification of the average plaque area was performed using the Image-Pro Plus software. The plaque area was presented as lesion % (% of whole aorta) (n = 6 sections per mice). Scale bars = 100.1 μ m. Magnification: $\times 100$. The data were analyzed by one-way ANOVA. Data represent mean $\pm$ SEM. n = 3 mice for each group. [#]P < 0.05 versus control; ^{*}P < 0.05 versus HHcy. Control, standard mouse diet group; HHcy, 1.5% methionine group, HHcy+Telmi: HHcy + telmisartan treatment group.

Figure 2

Hcy accelerates atherosclerosis formation (a) Representative Oil Red O staining of the thoracic aorta root sections. (b) Quantification of the average plaque area was performed using the Image-Pro Plus software. The plaque area was presented as lesion % (% of whole aorta) (n = 6 sections per mice). Scale bars = 100.1 μ m. Magnification: $\times 100$. The data were analyzed by one-way ANOVA. Data represent mean $\pm$ SEM. n = 3 mice for each group. [#]P < 0.05 versus control; ^{*}P < 0.05 versus HHcy. Control, standard mouse diet group; HHcy, 1.5% methionine group, HHcy+Telmi: HHcy + telmisartan treatment group.

Figure 2

HHcy increases features of plaque instability (a) Representative H&E staining. Magnification: $\times 200$. Scale bars = 100.1 μm . (b) Representative Masson staining. Collagen fibers showed as blue. Magnification: $\times 200$. Scale bars = 100 μm . (c) Total collagen fibers quantification. The percent of the positively stained area to the measured visual field area was reported, collagen content analysis was carried out using ImageJ software. Values represent mean $\pm$ SEM, $n = 3$ mice for each group. The data were analyzed by one-way ANOVA. ###P < 0.001 versus control; ***P < 0.001 versus HHcy. Control, standard mouse diet group; HHcy, 1.5% methionine group, HHcy+Telmi: HHcy + telmisartan treatment group.

Figure 3

HHcy promoted inflammation of plaques in ApoE^{-/-} male mice (a) Immunohistochemical staining shows MCP-1, IL-6, and Mac3 expression in each group. Scale bar = 100µm. Magnification: ×100. (b) Quantitative analysis of MCP-1, IL-6, and Mac-3 infiltration in atherosclerotic plaques of aortic roots. n = 3 mice for each group. Data represent mean ± SEM. The data were analyzed by one-way ANOVA. [#]P < 0.05 versus control; ^{*}P < 0.05 versus HHcy. Control, standard mouse diet group; HHcy, 1.5% methionine group, HHcy+Telmi: HHcy + telmisartan treatment group; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6; Mac3, macrophages.

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Figure 4

AT1R mediates HHcy-promoted inflammation of aortic adventitia in ApoE^{-/-} male mice (a) Representative immunohistochemistry staining of IL-6, MCP-1, and Mac3 infiltration in aortic adventitia in ApoE^{-/-} male mice. (b) Quantitative analysis of MCP-1, IL-6, and Mac3 infiltration in aortic adventitia. Scale bar = 100 μ m. Magnification: $\times 200$ n = 3 mice for each group. Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. #P < 0.05 versus control; *P < 0.05 versus HHcy. Control, standard mouse diet group; HHcy, 1.5% methionine group; HHcy+Telmi: HHcy + telmisartan treatment group.

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Figure 5

AT1R mediates HHcy-promoted inflammation of aortic adventitia in ApoE^{-/-} male mice (a) Representative immunohistochemistry staining of IL-6, MCP-1, and Mac3 infiltration in aortic adventitia in ApoE^{-/-} male mice. (b) Quantitative analysis of MCP-1, IL-6, and Mac3 infiltration in aortic adventitia. Scale bar = 100 μ m. Magnification: $\times 200$ n = 3 mice for each group. Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. #P < 0.05 versus control; *P < 0.05 versus HHcy. Control, standard mouse diet group; HHcy, 1.5% methionine group; HHcy+Telmi: HHcy + telmisartan treatment group.

Figure 5

HHcy induces AFs migration and invasion Representative immunofluorescence staining. Control, few ER-TR7 in adventitia of aorta root; HHcy, more ER-TR7 in the whole aortic wall and plaque; HHcy + Telmi, ER-TR7 decreased after telmisartan treatment and was mainly expressed in adventitia of aorta. Scale bar = 100µm. Magnification: ×100. Control, standard mouse diet group; HHcy, 1.5% methionine group; HHcy+Telmi, HHcy + telmisartan treatment group.

Figure 6

HHcy induces inflammation in AFs (a) The concentration of H₂O₂ in the cell supernatant was measured by a hydrogen peroxide kit. (b) Fluorescence microscopy showed that the green fluorescence detected by the ROS probe DCFH-DA was brighter in the Hcy group than the control group, when the cells treated with 200 µmol/L Hcy were incubated for 48 hours. (c) Western blot indicated IL-6 levels in cells treated with

200 $\mu\text{mol/L}$ Hcy in different periods of time. (d) Densitometric analysis of IL-6 was performed against GAPDH ($n = 3$). Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. * $P < 0.05$ versus 0 hour. ** $P < 0.01$ versus 0 hour. Figure b: Control, untreated cells; Hcy, cells treated with 200 $\mu\text{mol/L}$ Hcy, h: hour.

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HHcy induces AFs migration and invasion Representative immunofluorescence staining. Control, few ER-TR7 in adventitia of aorta root; HHcy, more ER-TR7 in the whole aortic wall and plaque; HHcy + Telmi, ER-TR7 decreased after telmisartan treatment and was mainly expressed in adventitia of aorta. Scale bar = 100 μm . Magnification: $\times 100$. Control, standard mouse diet group; HHcy, 1.5% methionine group; HHcy+Telmi, HHcy + telmisartan treatment group.

Figure 7

Hcy induces MMP-9 expression in AFs (a) Western blot indicated MMP-9 levels in cells treated with 200 $\mu\text{mol/L}$ Hcy in different periods of time. (b) Densitometric analyses of MMP-9 was performed against GAPDH ($n = 3$). Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. * $P < 0.05$ versus 0 hour; ** $P < 0.01$ versus 0 hour, h: hour.

Figure 7

HHcy induces inflammation in AFs (a) The concentration of H_2O_2 in the cell supernatant was measured by a hydrogen peroxide kit. (b) Fluorescence microscopy showed that the green fluorescence detected by the ROS probe DCFH-DA was brighter in the Hcy group than the control group, when the cells treated with 200 $\mu\text{mol/L}$ Hcy were incubated for 48 hours. (c) Western blot indicated IL-6 levels in cells treated with 200 $\mu\text{mol/L}$ Hcy in different periods of time. (d) Densitometric analysis of IL-6 was performed against GAPDH ($n = 3$). Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. * $P < 0.05$ versus 0 hour. ** $P < 0.01$ versus 0 hour. Figure b: Control, untreated cells; Hcy, cells treated with 200 $\mu\text{mol/L}$ Hcy, h: hour.

Figure 7

Hcy induces MMP-9 expression in AFs (a) Western blot indicated MMP-9 levels in cells treated with 200 $\mu\text{mol/L}$ Hcy in different periods of time. (b) Densitometric analyses of MMP-9 was performed against GAPDH ($n = 3$). Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. * $P < 0.05$ versus 0 hour; ** $P < 0.01$ versus 0 hour, h: hour.

Figure 8

Hcy induces AFs migration and invasion via AT1R (a) Cell viability was determined by Cell Counting Kit-8. Cells were treated with Hcy (0, 50, 100, 200, and 400 $\mu\text{mol/L}$) for 48 hours. (b) Cells were scratched and

treated with 200 $\mu\text{mol/L}$ Hcy for 48 hours. An image of the wound closure area. Scale bars = 200 μm , magnification: $\times 100$. (c) Quantification of the wound closure area was performed using the Image-Pro Plus software. (d) The cells (1×10^5 cells/well) were seeded in invasion chambers after Hcy treatment for 48 hours. Cells were stained with crystal violet. Scale bars = 100.1 μm , magnification: $\times 40$. (e) The quantification of invasion. $n = 3$. Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA if the data that failed to pass the normality test were analyzed by nonparametric test. # $P < 0.05$ versus control; * $P < 0.05$ versus Hcy; ** $P < 0.01$. Control, untreated cells; Hcy, cells treated with 200 $\mu\text{mol/L}$ Hcy; Hcy+Telmi: telmisartan pretreatment group.

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Figure 9

Hcy induces AT1R expression in AFs (a) Western blot indicated AT1R levels in AFs treated with 200 $\mu\text{mol/L}$ Hcy in different periods of time ($n = 3$). (b) Western blot indicated that AT1R levels in AFs were treated with various doses of Hcy (0 to 400 $\mu\text{mol/L}$) for 48 hours ($n = 3$). (c, d) Densitometric analyses of AT1R were performed against GAPDH. Data represent mean $\pm$ SEM. The data were analyzed by one-way ANOVA. $**P < 0.01$ compared with 0 or control. h: hour. (e) Fluorescence microscopy showed that the green fluorescence detected at 48 hours incubation was much brighter in the Hcy group than in the control group. Scale bar = 100 μm , magnification: $\times 100$ or $\times 200$.

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Figure 11

Hcy activates the AT1R downstream G protein-dependent signaling pathways in AFs (a–c) Representative western blots and quantification of phosphorylated and total PKC and ERK1/2 in Hcy (200 μ mol/L)-treated AFs with or without telmisartan (1 mmol/L) pretreatment for 60 minutes. n = 3. Data represent mean $\pm$ SEM. The data were analyzed by repeated measurement ANOVA. *P < 0.05, **P < 0.01 intragroup comparison; #P < 0.05, ##P < 0.01 between groups.

Figure 11

Expression of target protein in HEK293A cells (a) Agarose gel electrophoresis of plasmid DNA showing representative patterns of empty plasmid and overexpression of plasmid. (b) Expression of green fluorescent protein (EGFP). HEK293A cells transfected with overexpression of plasmid. Scale bar = 100

μm. (c–d) Western blot indicated AT1R levels in HEK293A cells and densitometric analyses. n = 3. Data represent mean ± SEM. The data were analyzed by one-way ANOVA. *P < 0.05 versus control group. Control, HEK293A cells; Over, overexpression of plasmid; Empty, empty plasmid HEK293A cells.

Figure 12

Hcy activates the AT1R downstream G protein-dependent signaling pathways in HEK293A cells. (a–c) Representative western blots and quantification of phosphorylated and total PKC and ERK1/2 in Hcy

(200 μ mol/L)-treated HEK293A cells (transfected with human AT1R) with or without telmisartan (1 mmol/L) pretreatment for 60 minutes. n = 3. Data represent mean $\pm$ SEM. The data were analyzed by repeated measurement ANOVA. *P < 0.05, **P < 0.01 intragroup comparison; #P < 0.05, ##P < 0.01 between groups.

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