Renin and (pro)renin receptor induced vascular smooth muscle cell proliferation and neointimal hyperplasia by activating oxidative stress and inflammation

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Research Article

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Abstract

Background

Numerous studies have shown that the proliferation and migration of vascular smooth muscle cells (VSMCs) were the pathological basis of restenosis occurrence. And Renin and prorenin could promote the proliferation of VSMCs through (pro)renin receptor ((P)RR). Therefore, this study aimed to observe the role of (P)RR in the proliferation of VSMCs in vitro and neointimal hyperplasia in the arterial injury model.

Methods

Cell proliferation was measured by CCK-8 and flow cytometry; RT-PCR and WB assays were used to detect the expressions of cyclin D1, PCNA, (P)RR, NOX1, and PI3K/AKT signal proteins; immunofluorescent staining was conducted to measure the expression of (P)RR; the levels of renin, PDGF-BB, inflammatory factors, and oxidative stress were determined by ELISA; pathological change was observed by HE staining.

Results

In this study, we demonstrated that renin could promote the proliferation of rat VSMCs by enhanced cell viability and cell cycle proteins, but silencing (P)RR restrained this. Then, we found that renin could enhance the levels of NOX1-mediated oxidative stress and inflammation by activating ERK1/2-AKT pathway in vitro. Similarly, inhibition of (P)RR resulted in opposite phenomenon. Importantly, inhibition of (P)RR could inhibit the neointimal hyperplasia in vivo after common carotid artery injury through restraining NOX1-mediated oxidative stress by down-regulating ERK1/2-AKT pathway.

Conclusion

We concluded that renin and (P)RR induced vascular smooth muscle cell proliferation and neointimal hyperplasia by activating oxidative stress, inflammation and ERK1/2-AKT pathway in an angiotensin (Ang) independent manner.

Introduction

The technique of percutaneous coronary intervention (PCI) was regarded as one of the useful treatments for coronary heart disease (CHD) with atherosclerosis, but approximately 30%-50% occurred vascular restenosis (RS) in half a year after PCI [1, 2]. Therefore, studying the mechanism of vascular neointimal hyperplasia after injury was of great significance for finding effective intervention targets. Among them, neointimal hyperplasia and vascular remodeling were important pathological mechanisms of RS, and vascular smooth muscle cells (VSMCs) played an indispensable role in this process [3, 4].
The renin-angiotensin system (RAS) was an important system for the body to maintain normal blood pressure, water and electrolyte balance, and cardiovascular homeostasis [5]. In recent years, with the deepening of research, we have a more comprehensive understanding of RAS. In 2002, Nguyen firstly discovered the renin (pro) receptor [(pro)renin receptor, (P)RR] and the discovery of this gave people a better understanding of the RAS system [6]. The function of renin-specific receptors mainly has two aspects: On the one hand, it produced angiotensin II (Ang II)-dependent effects. [7, 8]. On the other hand, it produced a Ang II-independent effect. After prorenin bound to renin-specific receptors, it activated the MAPK-ERK1/2 signal transduction pathway and further promoted the expressions of transforming growth factor-β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1). And this accelerated end-organ fibrosis [9]. Studies have shown that the combination of prorenin and (P)RR could promote renal fibrosis and myocardial fibrosis in a Ang II-independent manner [10, 11].

VSMCs have been confirmed to have prorenin receptors, and renin and prorenin could promote the proliferation of vascular smooth muscle cells through (P)RR [12]. Therefore, we inferred that the receptor-associated prorenin system (RAPS) played a very important role in the occurrence of RS. In 2007, researchers have found that prorenin could promote the proliferation of VSMCs [13]. However, it was not clear whether prorenin promoted the proliferation of VSMCs through the (P)RR-mediated Ang II-independent way.

In this study, we investigated whether (P)RR could mediate inflammation, oxidative stress and ERK1/2-AKT pathway, which led to proliferation of VSMCs in vitro and vascular neointimal hyperplasia in vivo in a (P)RR-mediated Ang II-independent manner.

Materials And Methods

Balloon injury model and treatment

The healthy male SD rats were provided by the Experimental Animal Center of Shanxi Medical University, weighing 250-300g. Standard feed and free drinking water. All animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals. 2% sodium pentobarbital (50mg/kg) was used to inject intraperitoneally for anesthesia. Incising the skin and subcutaneous tissue along the anterior midline of the neck, and exposing the left common carotid artery and internal and external carotid arteries in the anterior cervical triangle. Then, the distal end of the external carotid artery was ligated; the blood flow at the proximal end of the common carotid artery was closed with a vascular clip. Incising a wedge-shaped inside the distal end of the external carotid artery, inserting a 1.5F balloon catheter from the external carotid artery to the beginning of the common carotid artery, and inflating the balloon to block the blood flow for 30 seconds, then slowly pulling the balloon back to the bifurcation of the internal and external carotid arteries for three times, and withdrawing the balloon. At last, the outside of the neck arteries was ligated, subcutaneous tissue and skin was sutured layer by layer.
The low-HRP (L-HRP) and high-HRP (H-HRP) groups were treated with 10 μg/kg and 100 μg/kg HRP (inhibitor of (P)RR, glbiochem, shanghai) respectively by tail vein injection. The positive control group was intragastric administration with 100 mg/kg Losartan (Los, antagonist of angiotensin I receptor, Sigma). All these treatments lasted for 17 consecutive days from 3 days before surgery.

Cell culture and treatment

Vascular smooth muscle cells (VSMCs) of rats, purchased from Procell (Wuhan, China), were cultured in Dulbecco's modified Eagle's (DMEM) medium (Gibco, USA) containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin under 5% CO₂ at 37 °C. Cells were treated with different concentration of renin (Sigma-Aldrich, USA), 10⁻⁶ mol/L Los, 10⁻⁵ mol/L PD123319 (antagonist of angiotensin II receptor, Sigma), 10nmol/L platelet-derived growth factor-BB (PDGF-BB, PeproTech), or 10⁻⁵ mol/L diphenyleneiodonium chloride (DPI, inhibitor of NADPH, Sigma-Aldrich, USA). Renin, PDGF-BB, and DPI were added after cells were treated with Los and PD123319 for 30 min.

Construction of miRNA interference plasmid of (P)RR

Four pairs of miRNAs and negative control (NC) sequence were designed and synthesized according to the gene sequence (GenBank accession number: AB188298). Then, miRNAs and NC were all cloned into pcDNA™6.2-GW/EmGFP vector (Invitrogen, USA) to determine the most effective miRNA. Cells were transfected with miRNA-(P)RR using Lipofectamine™2000 reagent (Invitrogen, USA).

Cell counting kit-8 assay

Collecting the logarithmic phase VSMCs, and adjusting the cell suspension concentration to 1×10⁴/well. Cells treated or transfected with different reagent were incubated with 5% CO₂ at 37°C overnight until the cell monolayer covered the bottom of the 96-well plate. Then, cells in each well were added 10 μl CCK-8 (Solarbio, China) solution and continued to culture for another 4 h. Microplate reader was used to detect the optical density of each well at 450 nm wavelength.

Detection of cell cycle

Cells were seeded into 6-well plates at a density of 1×10⁶/well and cultured overnight. Then, cells were treated or transfected with corresponding reagent and continue culturing for another 48 h at 37°C, 5% CO₂ incubator. Collecting the cells after centrifugation, discarding the supernatant, washing the cells three times with pre-cooled PBS, adding pre-cooled 70% ethanol, and fixing the cells at 4°C overnight. Next, cells were collected, washed with PBS, and added into RNAase for incubation at 37°C, 30 min. Then, cells
were stained with 50 μl PI and incubated at 4°C for 30 min in dark. Lastly, cell cycle was detected by flow cytometry (FCM) and analyzed by ModFit software.

**Measurement of MDA and SOD**

VMSCs were seeded into plates and cultured overnight. Then cells were collected for experiments. The production of malondialdehyde (MDA) and antioxidant enzymes superoxide dismutase (SOD) in VMSCs were examined using the MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and Superoxide Dismutase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively on the basis of the manufacturer’s specification.

**Measurement of ROS**

Reactive Oxygen Species Assay Kit (Beyotime, China) was used in this experiment. Briefly, VMSCs were inoculated in a 6-well plate (1×10^4 cells/well), cultured overnight until the cells were fused to 70%-80%, and performed the experiment. The positive control group was treated with H_2O_2 at a final concentration of 100 μM/L for 1 h. At the time point, the culture medium was discarded and rinsed three times with PBS at room temperature. Then, cells were added the DCFH-DA fluorescent probe (diluted probe without serum DMEM) at the final concentration of 10 μM/L, incubated at 37°C in the dark for 20 min and rinsed three times with PBS for observing the intracellular fluorescence under a microscope. Collecting and transferring the cell suspension into a flow tube, adding 400 μl PBS, and measuring the fluorescence intensity with a flow cytometer to indirectly present the level of reactive oxygen species (ROS).

**Immunofluorescence staining assay**

VMSCs in a 12-well plate were treated with different reagents for 24 h. Then the cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 15 min, and then washed with PBS for three times. 5% skimmed milk in PBS was then added to the desired wells for 30 min at 37°C. Then, the cells were washed and stained with primary antibody (P)RR (Sigma-Aldrich, USA) and alpha-smooth muscle actin (α-SMA) (Abcam, UK) at 4°C overnight. After six times washing, the cells were incubated with fluorescein isocyanate (FITC)-conjugated goat anti-mouse (KPL, USA) as secondary antibody at 37°C for 1 h. DAPI (Solarbio, China) was added for incubation in dark for 5min, and the excess DAPI was washed by PBS for 5 min. Finally, images were acquired using fluorescent microscope (Nikon, Tokyo, Japan) at 400× magnification.

**H&E staining**

Common carotid artery tissues from each group were fixed in 4% paraformaldehyde at room temperature, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E). The vascular intimal hyperplasia and histopathological change were observed under the light microscope.
(Olympus, Tokyo, Japan) at 100× and 400× magnification and quantified by the intima/media area ratio (I/M).

**Enzyme-linked immunosorbent assay (ELISA)**

Arterial blood from mice and VMSCs in each group were collected, the levels of renin (Abcam, UK) and PDGF-BB (PeproTech, China) in mice and proinflammatory cytokines including tumor necrosis factor-α (TNF-α, Beyotime, China), interleukin-6 (IL-6, Abcam, UK), interleukin-1β (IL-1β, Beyotime, China), and inducible nitric oxide synthase (iNOS, Abcam, UK) were determined by ELISA based on the manufacturer's instructions. The OD value of each well was immediately read at 450 nm.

**Real time-PCR**

Total RNAs from common carotid artery wall tissues or the VMSCs were isolated using TRizol reagent according to the manufacturer's protocol (Invitrogen, Thermo Fisher Scientific). The expressions of NOX1, cyclin D1, proliferating cell nuclear antigen (PCNA) in tissues and (P)RR, NOX1, cyclin D1, PCNA in cells were determined using the SYBR Premix Ex TaqTM II Kit (Takara, Tokyo, Japan) on the basis of the manufacturer's protocol. β-actin served as a control. The 2^−ΔΔCt method was used to quantify the relative expression levels of (P)RR, NOX1, cyclin D1, and PCNA.

**Western blot assay**

To confirm the expression levels of ERK1/2, p-ERK1/2, AKT, p-AKT, cyclin D1, PCNA, (P)RR, and NOX1 in common carotid artery wall tissues and VMSCs, HTR8/SVneo cells were lysed with RIPA buffer. Lysates were separated on 10% SDS-polyacrylamide gel and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 5% non-fat milk for 1 h at room temperature, and membranes were incubated with corresponding primary antibodies at 4ºC overnight following incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Protein expressions were detected with high sensitivity ECL chemiluminescence detection kit (Vazyme, China). The bands intensity was expressed as fold change by normalizing the data to the values of β-actin using ImageJ software.

**Statistical analysis**

Statistical analysis was performed by SPSS22.0 and GraphPad Prism 9 software. Data were showed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) or two-way ANOVA were used to compare the difference among multiple groups followed by post-hoc tests. p<0.05 was thought to be statistically significant.

**Results**

**Renin improved proliferation of VSMCs**
To demonstrate whether renin improve VSMCs proliferation, VSMCs were treated with Los and PD123319 or further supplemented with different concentration of renin after 30 min. CCK-8 assay showed that the OD value in different concentration of renin increased markedly in a concentration-dependent way compared with the control group (Fig. 1A). Then the results of FCM demonstrated that the composition ratio of G1 phase decreased in the VSMCs with the up-regulation of renin concentration, but the S phase and the proliferation index were increased (Fig. 1B, C). RT-PCR and WB assays indicated that the mRNA and protein levels of cyclin D1 and PCNA were both markedly up-regulated along with increasing concentration of renin (Fig. 1D, E). Furthermore, to observe the expression of (P)RR following renin treatment, the immunofluorescence staining assay was conducted. Results showed that high concentration of renin had enhanced mean fluorescence intensity (MFI) of (P)RR (Fig. 1F). These data suggested that the level of (P)RR was increased after renin treatment and renin could promote the proliferation of VSMCs in a concentration-dependent and Ang-independent manner.

**Silencing (P)RR restrained VSMCs proliferation**

To figure out whether the function of renin was associated with (P)RR, we designed and synthesized four pairs of miRNAs and NC sequence to silence (P)RR, and named as RNAi-(P)RR. The sequences were shown in Table 1. RT-PCR and WB demonstrated that the most effective miRNA was RNAi-(P)RR-2 (Fig. 2A, B). Among the growth factors released by damaged cells in the early stage of arterial injury, PDGF-BB could promote the proliferation of VSMCs [14]. Therefore, addition of PDGF-BB could serve as a positive control. Firstly, the mRNA and protein levels of (P)RR were both significantly down-regulated by RNAi-(P)RR treatment through RT-PCR and WB assays (Fig. 2C, D). Then, the VSMCs proliferation was indicated by CCK-8. Results showed that compared with the PDGF-BB or renin treatment group, the OD values of VSMCs were significantly decreased after treatment with RNAi-(P)RR (Fig. 2E). Similarly, in the miRNA-(P)RR+PDGF-BB or miRNA-(P)RR+renin group, the constituent ratio of G1 phase was increased, while the constituent ratio of S phase and proliferation index were decreased (Fig. 2F, G). This was further proved by the markedly reduced mRNA and protein levels of cyclinD1 and PCNA in RNAi-(P)RR-treated groups (Fig. 2H, I). These results above suggested that the pro-proliferation function of renin in VSMCs was mediated by (P)RR in a Ang-independent way.

**Renin induced VSMCs proliferation via activating oxidative stress and inflammatory response**

For illustrating the mechanism of renin, we focused on the oxidative stress and inflammatory response. FCM and ELISA manifested that renin treatment could significantly enhance the levels of ROS and MDA, while reduce SOD expression (Fig. 3A, B). However, VSMCs treated with DPI (NADPH oxidase inhibitor) down-regulated or increased the expression level of ROS, MDA or SOD compared to control group, and renin+DPI treatment could revert the function of renin (Fig. 3A, B). Additionally, the levels of pro-inflammatory cytokines including IL-6, TNF-α, IL-1β, and iNOS were all markedly increased by renin
treatment compared with control group, but decreased after treated with DPI (Fig. 3C). These suggested that renin treatment could activate oxidative stress and inflammation. Next, FCM indicated that the renin+DPI group had an increase in G1 phase constituent ratio, while a decrease in S phase constituent ratio and proliferation index compared with the renin group (Fig. 3D, E). The same phenomenon was observed by the reduced mRNA and protein levels of cyclinD1 and PCNA in renin+DPI group in comparison with renin group (Fig. 3F, G). Lastly, the protein levels of p-ERK1/2 and p-AKT were both significantly increased in renin-treated group and partly reduced by DPI supplement (Fig. 3H). These data indicated that renin could enhance VSMCs proliferation through up-regulating oxidative stress and inflammatory response, and ERK1/2-AKT signaling pathway may be involved in this process.

Silencing (P)RR decreased the levels of oxidative stress and inflammation

Considering that silencing (P)RR could revert the pro-proliferation function of renin, we then explored whether silencing (P)RR could reverse oxidative stress and inflammation induced by renin. Results showed markedly increased MDA and decreased SOD levels in VSMCs supplemented with PDGF-BB or renin, but the MDA and SOD levels were significantly reduced and enhance by RNAi-(P)RR treatment, respectively (Fig. 4A, B). Then, the IL-6, TNF-α, IL-1β, and iNOS expressions were all remarkably enhanced in PDGF-BB or renin group than control group, but decreased after addition of RNAi-(P)RR (Fig. 4C). It was found that ROS induced by NOX1 could promote the proliferation of vascular smooth muscle cells [15], so the expression of NOX1 was also determined. Results showed by RT-PCR and WB indicated that the mRNA and protein levels were both markedly up-regulated in PDGF-BB or renin group, in contrast, the over-expressions of NOX1 could be reversed by RNAi-(P)RR (Fig. 4D, E). Interestingly, we found that RNAi-(P)RR could restrain the ERK1/2-AKT pathway through inhibiting the expressions of p-ERK1/2 and p-AKT (Fig. 4F). These data indicated that (P)RR, which regulated by renin, could promote the proliferation of VSMCs by regulating NOX1-mediated oxidative stress and inflammation to activate ERK1/2-AKT signaling pathway.

Inhibition of (P)RR restrained neointimal hyperplasia after common carotid artery injury of rats

In order to elucidate the function of (P)RR in vivo, the balloon injury model was constructed to present common carotid artery injury. Primarily, HE staining showed that the neointimal hyperplasia was distinct after injury, resulting in obviously and slightly thickened medial layer in injury and L-HRP group, respectively. However, these phenomena were alleviated after H-HRP and Losartan treatment (Fig. 5A). Importantly, the I/M ratio in the low HRP (L-HRP) group was not significantly different from those in the injury group, but Losartan and high HRP (H-HRP) treatment showed a slight neointimal hyperplasia and markedly reduced I/M ratio than the injury group (Fig. 5B). Furthermore, the mRNA and protein expressions of Cyclin D1 and PCNA in vascular wall of injury group were both enhanced significantly than
sham group, decreased markedly in Losartan and H-HRP group after injury, but no significant difference was observed in L-HRP group compared with injury group (Fig. 5C, D). In addition, ELISA assay indicated that the levels of renin and PDGF-BB in arterial blood were both increased after injury, but reduced by Losartan and H-HRP treatment, with no marked difference in L-HRP group (Fig. 5E), further suggesting the pro-proliferation effect of renin. These results manifested that (P)RR could improve neointimal hyperplasia after common carotid artery injury of rats.

Inhibition of (P)RR restrained NOX1-mediated oxidative stress by down-regulating ERK1/2-AKT pathway

In the following, we aimed to explore whether the specific mechanism of (P)RR was related to oxidative stress and ERK1/2-AKT pathway. By detection of SOD and MDA levels in vascular wall, we found that the levels of MDA and SOD in the injury group were obviously higher and lower compared with the sham operation group. In contrast, the MDA and SOD levels in Losartan and the H-HRP groups were remarkably reduced and increased, while the L-HRP group had no significant change (Fig. 6A, B). Next, results by RT-PCR and WB indicated that the mRNA and protein levels of NOX1 were both markedly increased in the injury group, but decreased by Losartan and H-HRP treatment, while the L-HRP group had no significant difference (Fig. 6C, D). Lastly, WB assay showed that the expressions of p-ERK1/2 and p-AKT were enhanced after injury and decreased by Losartan and H-HRP groups, but no marked difference was observed in L-HRP group compared with injury group (Fig. 6E). These data demonstrated that inhibition of (P)RR restrained NOX1-mediated oxidative stress by down-regulating ERK1/2-AKT pathway.

Discussion

In this study, we successfully constructed and screened the miRNA interference plasmid against the rat (P)RR gene and demonstrated that renin could promote the proliferation of rat VSMCs through (P)RR in Ang-II-independent manner and this function of renin was related to enhanced NOX1-mediated oxidative stress and inflammation by activating ERK1/2-AKT pathway in vitro. Similarly, inhibition of (P)RR could inhibit the neointimal hyperplasia in vivo after common carotid artery injury through restraining NOX1-mediated oxidative stress by down-regulating ERK1/2-AKT pathway.

At present, it was widely believed that the migration and proliferation of VSMCs after vascular endothelial injury were the pathological basis of atherosclerosis and RS after PCI [16, 17]. In many pathological conditions, the growth factors increased, and then stimulated the signal transduction network to promote the proliferation of VSMCs, leading to a series of pathological changes in the blood vessel wall [18]. RAS served as a classic cardiovascular regulation system, it played an indispensable role in the proliferation of VSMCs [19, 20]. As a new member of the RAS system, (P)RR played an essential role in the pathogenesis of cardiovascular diseases and diabetic nephropathy [21]. In the past, it was well believed that renin must rely on Ang II to exert its biological activity. However, in recent years, many studies have
shown that the biological role of renin precursor may not depend on traditional RAS activation, but related to (P)RR [22].

Researchers have confirmed that the prorenin could promote the increase and hypertrophy of VSMCs [23, 24]. However, whether renin promoted the proliferation of VSMCs through (P)RR-mediated Ang II-independent way remained unclear. Moreover, the division and proliferation of VMSCs was mainly regulated by the eukaryotic cell cycle [25]. VSMCs in cell cycle gone through four stages in turn: G1, S, G2, and M. Specifically, cyclin D 1 was an important cyclin that accelerated cells to the S phase through the G1/S restriction point and played a positive regulatory role on cell proliferation [26]. In addition, PCNA was an intranuclear polypeptide synthesized or expressed only in proliferating cells. It increased in the late G1 phase of the cell cycle, reached a peak in the S phase, and decreased in the G2 and M phases. It was a specific marker of the S phase, so it could be used as an indicator to evaluate cell proliferation [27]. In this experiment, VSMCs were treated with different concentrations of renin, and transfected with the RNAi-(P)RR plasmid to explore the effect of (P)RR. Based on these results, we found that the level of (P)RR had a marked increase after renin treatment. And renin could significantly promote the proliferation of VSMCs, S phase composition ratio, proliferation index, and expressions of cyclin D1 and PCNA in a concentration-dependent and Ang II-independent manner. However, inhibition of (P)RR could revert the pro-proliferation of renin, suggesting that the pro-proliferation function of renin in VSMCs was mediated by (P)RR in a Ang II-independent way.

Local vascular damage caused by oxidative stress has been considered to be one of the important factors for RS after PCI. After local vascular injury, the concentration of oxidative active substances increased significantly, which played an important role in stimulating the proliferation of VSMCs [28]. Recent studies have shown that NADPH oxidase was the main source of ROS in the vascular system. VMSCs [29] also have a small amount of ROS continuously generated when they are not stimulated, and ROS generation was significantly increased under the action of certain stimulants. The application of NADPH oxidase inhibitor, for example, DPI, has confirmed that ROS is generated by NADPH oxidase. Besides, NOX1, a member of NOX family, was up-regulated in rat carotid arteries after balloon injury [30]. More importantly, inflammatory process remained to be a predominant major cause of CHD with atherosclerosis worldwide [31]. Primary or upstream pro-inflammatory cytokines such as IL-1β or TNF-α were secreted in all phases of atherosclerotic lesion progression and induced expression of “messenger” or secondary signaling cytokines such as IL-6 [32]. Furthermore, activation of the iNOS could enhance inflammatory processes within the vascular wall and contributed to atherosclerosis progression [33]. ROS participated in a variety of signal transduction pathways, including cell membrane receptors or channels to various protein kinases and nuclear transcription factors [34]. Such as activating the extracellular signal-regulated kinase1/2 (ERK1/2), mitogen-activated protein kinase (MAPK) activity, and also could strengthen the signal transduction of JAK/STAT [35, 36], promoting cell proliferation and vascular remodeling. Our research found that renin treatment could significantly enhance the levels of ROS and MDA, while reduce SOD expression, but DPI treatment could reverse this. Additionally, the levels of pro-inflammatory cytokines including IL-6, TNF-α, IL-1β, and iNOS were all increased by renin treatment, but decreased after treated with DPI. Next, FCM and WB assays indicated that the renin promoted the
proliferation of VSMCs, while DPI inhibited this. Besides, the protein levels of p-ERK1/2 and p-AKT were increased in renin-treated group and partly reduced by DPI supplement. These results suggested that renin promoted VSMCs proliferation by up-regulating oxidative stress, inflammatory response, and ERK1/2-AKT signaling pathway.

Previous experimental results have shown that (P)RR played an important role in the proliferation of VSMCs, while the proliferation and differentiation of VSMCs were the main pathological basis of RS. Therefore, we further investigated the role and mechanism of (P)RR in the restenosis of carotid artery injury model. Studies have shown that the proliferation and migration of VSMCs after balloon injury are key factors in the formation of restenosis after PCI [37, 38]. After arterial injury, VSMCs migrated from the medial membrane to the luminal surface through the internal elastic plate. After migration to the luminal surface, VSMCs began to proliferate and form new intima. Hence, proliferation and dedifferentiation of VSMCs play an important role in the neointimal hyperplasia. Earlier study has identified that Losartan, a nonpeptide Ang II receptor antagonist, inhibited neointima formation following balloon injury to rat carotid arteries [39]. Handle region peptides (HRP) could mimic the stalk region and thus replace the binding of prorenin to (P)RR, so HRP was also known as a prorenin receptor blocker [40]. Therefore, losartan and HRP were used in this study to observe the role of (P)RR in restenosis after carotid artery injury. It has been reported that superoxide anion (O$_2^-$) and lipid peroxidation products can be produced by local vascular wall cells in a very short time after PTCA, and the MDA level in coronary veins was also significantly higher than that before PTCA [41].

In this research, HE staining and I/M ratio showed that the neointimal hyperplasia was distinct after injury, but these phenomena were alleviated after H-HRP and Losartan treatment. Furthermore, the expressions of Cyclin D1 and PCNA in injury group were both enhanced significantly than sham group, decreased markedly in Losartan and H-HRP group. In addition, ELISA assay indicated that the levels of renin and PDGF-BB in arterial blood were both increased after injury, but reduced by Losartan and H-HRP treatment, further suggesting the pro-proliferation effect of renin. Next, the data showed that the MDA, NOX1, p-ERK1/2 and p-AKT levels of the injury group was obviously higher and lower after Losartan and H-HRP treatment, while the expression of SOD presented the opposite trend. These results manifested that (P)RR could improve neointimal hyperplasia after common carotid artery injury of rats by regulating NOX1-mediated oxidative stress and ERK1/2-AKT pathway.

**Conclusion**

In conclusion, our data indicated that renin and (P)RR could enhance VSMCs proliferation and neointimal hyperplasia through up-regulating oxidative stress, inflammatory response and ERK1/2-AKT signaling pathway in a Ang- independent manner. These results may provide a new perspective for targeted therapy of RS in coronary heart disease.

**Declarations**
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Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

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

Author Contributions

Chuanshi Xiao and Nana Zhang designed the experiments. Nana Zhang, Xiaosu Song and Yunfei Bian carried out the experiments. Rui Bai and Huiyu Yang performed the statistical analysis. Gang Wang and Hong Li drafted the manuscript. All authors gave final approval, agreed to be accountable for all aspects of work ensuring integrity and accuracy.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Tables

Table 1. miRNA oligomeric single-stranded DNA sequence of (P)RR

| Names of miRNA     | Sequence (5’to 3’)                                      |
|--------------------|--------------------------------------------------------|
| RNAi-(P)RR-1F      | TGCTGATAGGTGCCAGCACAGCGGCTTTTGGGCC                     |
|                    | ACTGACTGACGCGCTTTGCTGGCAACCTAT                        |
| RNAi-(P)RR-1R      | CCTGATAGGTGCCAGCAAGCGCTCTGAGTCAGTGG                   |
|                    | CCAAAAAGCGGCTTTGCTGGCAACCTAT                          |
| RNAi-(P)RR-2F      | TGCTGTGTTAGCTCTCAAGACAGTTTGCTGGCC                     |
|                    | ACTGACTGACCCCTTGCTGGACAGTCTAAAA                      |
| RNAi-(P)RR-2R      | CCTGTTTAGACTCTCAGAGAGGTCTGCTGGGCAACCTAT              |
| RNAi-(P)RR-3F      | TGCTGTCTACATGCTACCATAATGGATTGCTTTTGCC                |
|                    | CTGACTGACTACATTAGTGACAGTGAA                          |
| RNAi-(P)RR-3R      | CCTGTTGCTAAGCTACTAATGGATTGCTGAGTCAGTGG               |
|                    | CCAAAACTACCATTAGTGACAGTGAAC                         |
| RNAi-(P)RR-4F      | TGCTGTTCTGCTCAGTCTGGAAGGTCTGCTGAA                    |
|                    | ACTGACTGACTTTCTGAGACTGCAACCCGAA                     |
| RNAi-(P)RR-4R      | CCTGTTGCTGGAAGTGACTGTAAGGTCTGAGTCAGTGG              |
|                    | CCAAAACTTTCGAGAACCCTGCAACCAGAC                     |
| RNAi-(P)RR-NC-F    | TGCTGAAATGTACTGCGCCTGGAGACGTCTGTTTGCC               |
|                    | ACTGACTGACGTCTCCACGCAGTCAATT                        |
| RNAi-(P)RR-NC-R    | CCTGAAATGTACTGCGTGGAGACGTCTGAGTCAGTGG               |
|                    | CCAAAACGTCTCCACGCAGTCAGATTT                        |