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

Nana Zhang
First Hospital of Shanxi Medical University
Xiaosu Song
Second Hospital of Shanxi Medical University
Yunfei Bian
Second Hospital of Shanxi Medical University
Rui Bai
Second Hospital of Shanxi Medical University
Huiyu Yang
Second Hospital of Shanxi Medical University
Gang Wang
First Hospital of Shanxi Medical University
Hong Li
Second Hospital of Shanxi Medical University
Chuanshi Xiao (xiaochuanshi1955@163.com)
First Hospital of Shanxi Medical University https://orcid.org/0000-0003-0654-2997

Research Article

Keywords: Renin, (pro)renin receptor, VSMCs, proliferation, oxidative stress, inflammation

Posted Date: March 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-298545/v1

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% CO_2 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 1×10^4/well. Cells treated or transfected with different reagent were incubated with 5% CO_2 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 1×10^6/well and cultured overnight. Then, cells were treated or transfected with corresponding reagent and continue culturing for another 48 h at 37℃, 5% CO_2 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℃, 30 min. Then, cells

were stained with 50 μl PI and incubated at 4℃ 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 $2^{-\Delta\Delta Ct}$ 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 Ⅱ-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 Ⅱ-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 enhancive 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 D1and 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 □-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 □-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-egulated 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-Ⅱ independent manner. These results may provide a new perspective for targeted therapy of RS in coronary heart disease.

Declarations

Funding

This work was supported by Research Funding Project for Returned Overseas Students in Shanxi Province (No. 2011-110).

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

References

1. Weintraub WS (2007) The pathophysiology and burden of restenosis. Am J Cardiol 100 (5a):3k-9k. doi:10.1016/j.amjcard.2007.06.002
2. Nakatani M, Takeyama Y, Shibata M, Yorozuya M, Suzuki H, Koba S, Katagiri T (2003) Mechanisms of restenosis after coronary intervention: difference between plain old balloon angioplasty and stenting. Cardiovasc Pathol 12 (1):40-48. doi:10.1016/s1054-8807(02)00135-7
3. Chung IM, Gold HK, Schwartz SM, Ikari Y, Reidy MA, Wight TN (2002) Enhanced extracellular matrix accumulation in restenosis of coronary arteries after stent deployment. J Am Coll Cardiol 40 (12):2072-2081. doi:10.1016/s0735-1097(02)02598-6

4. Zargham R (2008) Preventing restenosis after angioplasty: a multistage approach. Clin Sci (Lond) 114 (4):257-264. doi:10.1042/cs20070228
5. Cosarderelioglu C, Nidadavolu LS, George CJ, Oh ES, Bennett DA, Walston JD, Abadir PM (2020) Brain Renin-Angiotensin System at the Intersect of Physical and Cognitive Frailty. Front Neurosci 14:586314. doi:10.3389/fnins.2020.586314
6. Nguyen G, Delarue F, Burcklé C, Bouzhir L, Giller T, Sraer JD (2002) Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. J Clin Invest 109 (11):1417-1427. doi:10.1172/jci14276
7. Shapiro BA, Olala L, Arun SN, Parker PM, George MV, Bollag WB (2010) Angiotensin II-activated protein kinase D mediates acute aldosterone secretion. Mol Cell Endocrinol 317 (1-2):99-105. doi:10.1016/j.mce.2009.11.017
8. Pereira LG, Arnoni CP, Maquigussa E, Cristovam PC, Dreyfuss J, Boim MA (2012) (Pro)renin receptor: another member of the system controlled by angiotensin II? J Renin Angiotensin Aldosterone Syst 13 (1):1-10. doi:10.1177/1470320311423280
9. Huang Y, Wongamorntham S, Kasting J, McQuillan D, Owens RT, Yu L, Noble NA, Border W (2006) Renin increases mesangial cell transforming growth factor-beta1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms. Kidney Int 69 (1):105-113. doi:10.1038/sj.ki.5000011
10. Li Z, Zhou L, Wang Y, Miao J, Hong X, Hou FF, Liu Y (2017) (Pro)renin Receptor Is an Amplifier of Wnt/β-Catenin Signaling in Kidney Injury and Fibrosis. J Am Soc Nephrol 28 (8):2393-2408. doi:10.1681/asn.2016070811
11. Zhou G, Wu J, Gu C, Wang B, Abel ED, Cheung AK, Huang Y (2018) Prorenin independently causes hypertension and renal and cardiac fibrosis in cyp1a1-prorenin transgenic rats. Clin Sci (Lond) 132 (12):1345-1363. doi:10.1042/cs20171659
12. Cousin C, Bracquart D, Contrepas A, Corvol P, Muller L, Nguyen G (2009) Soluble form of the (pro)renin receptor generated by intracellular cleavage by furin is secreted in plasma. Hypertension 53 (6):1077-1082. doi:10.1161/hypertensionaha.108.127258
13. Yoshikawa A, Aizaki Y, Kusano K, Kishi F, Susumu T, Iida S, Ishiura S, Nishimura S, Shichiri M, Senbonmatsu T (2011) The (pro)renin receptor is cleaved by ADAM19 in the Golgi leading to its secretion into extracellular space. Hypertens Res 34 (5):599-605. doi:10.1038/hr.2010.284
14. Abid MR, Yano K, Guo S, Patel VI, Shrikhande G, Spokes KC, Ferran C, Aird WC (2005) Forkhead transcription factors inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia. J Biol Chem 280 (33):29864-29873. doi:10.1074/jbc.M502149200
15. Lee MY, San Martin A, Mehta PK, Dikalova AE, Garrido AM, Datla SR, Lyons E, Krause KH, Banfi B, Lambeth JD, Lassègue B, Griendling KK (2009) Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. Arterioscler Thromb Vasc Biol 29 (4):480-487. doi:10.1161/atvbaha.108.181925

16. Marx SO, Totary-Jain H, Marks AR (2011) Vascular smooth muscle cell proliferation in restenosis. Circ Cardiovasc Interv 4 (1):104-111. doi:10.1161/circinterventions.110.957332
17. Basatemur GL, Jørgensen HF, Clarke MCH, Bennett MR, Mallat Z (2019) Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol 16 (12):727-744. doi:10.1038/s41569-019-0227-9
18. Rakesh K, Agrawal DK (2005) Cytokines and growth factors involved in apoptosis and proliferation of vascular smooth muscle cells. Int Immunopharmacol 5 (10):1487-1506. doi:10.1016/j.intimp.2005.05.003
19. Kumar R, Thomas CM, Yong QC, Chen W, Baker KM (2012) The intracrine renin-angiotensin system. Clin Sci (Lond) 123 (5):273-284. doi:10.1042/cs20120089
20. Friedrich EB, Clever YP, Wassmann S, Werner N, Böhm M, Nickenig G (2006) Role of integrin-linked kinase in vascular smooth muscle cells: regulation by statins and angiotensin II. Biochem Biophys Res Commun 349 (3):883-889. doi:10.1016/j.bbrc.2006.07.217
21. Cousin C, Bracquart D, Contrepas A, Nguyen G (2010) Potential role of the (pro)renin receptor in cardiovascular and kidney diseases. Journal of nephrology 23 (5):508-513
22. Peters J (2017) The (pro)renin receptor and its interaction partners. Pflugers Arch 469 (10):1245-1256. doi:10.1007/s00424-017-2005-z
23. Liu G, Hitomi H, Hosomi N, Shibayama Y, Nakano D, Kiyomoto H, Ma H, Yamaji Y, Kohno M, Ichihara A, Itoh H, Nishiyama A (2011) Prorenin induces vascular smooth muscle cell proliferation and hypertrophy via epidermal growth factor receptor-mediated extracellular signal-regulated kinase and Akt activation pathway. J Hypertens 29 (4):696-705. doi:10.1097/HJH.0b013e328343c62b
24. Sakoda M, Ichihara A, Kaneshiro Y, Takemitsu T, Nakazato Y, Nabi AH, Nakagawa T, Suzuki F, Inagami T, Itoh H (2007) (Pro)renin receptor-mediated activation of mitogen-activated protein kinases in human vascular smooth muscle cells. Hypertens Res 30 (11):1139-1146. doi:10.1291/hypres.30.1139
25. Wei GL, Krasinski K, Kearney M, Isner JM, Walsh K, Andrés V (1997) Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. Circ Res 80 (3):418-426
26. Stacey DW (2003) Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr Opin Cell Biol 15 (2):158-163. doi:10.1016/s0955-0674(03)00008-5
27. Strzalka W, Ziemienowicz A (2011) Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Ann Bot 107 (7):1127-1140. doi:10.1093/aob/mcq243
28. Kochiadakis GE, Arfanakis DA, Marketou ME, Skalidis EI, Igoumenidis NE, Nikitovic D, Giaouzaki A, Chlouverakis G, Vardas PE (2010) Oxidative stress changes after stent implantation: a randomized comparative study of sirolimus-eluting and bare metal stents. Int J Cardiol 142 (1):33-37. doi:10.1016/j.ijcard.2008.12.105
29. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 74 (6):1141-1148. doi:10.1161/01.res.74.6.1141

30. Szöcs K, Lassègue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK (2002) Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. Arterioscler Thromb Vasc Biol 22 (1):21-27. doi:10.1161/hq0102.102189
31. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jiménez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P (2017) Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation 135 (10):e146-e603. doi:10.1161/cir.0000000000000485
32. Libby P, Ridker PM, Hansson GK (2009) Inflammation in atherosclerosis: from pathophysiology to practice. J Am Coll Cardiol 54 (23):2129-2138. doi:10.1016/j.jacc.2009.09.009
33. Gliozzi M, Scicchitano M, Bosco F, Musolino V, Carresi C, Scarano F, Maiuolo J, Nucera S, Maretta A, Paone S, Mollace R, Ruga S, Zito MC, Macrì R, Oppedisano F, Palma E, Salvemini D, Muscoli C, Mollace V (2019) Modulation of Nitric Oxide Synthases by Oxidized LDLs: Role in Vascular Inflammation and Atherosclerosis Development. Int J Mol Sci 20 (13). doi:10.3390/ijms20133294
34. Griendling KK, Sorescu D, Lassègue B, Ushio-Fukai M (2000) Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol 20 (10):2175-2183. doi:10.1161/01.atv.20.10.2175
35. Yoshizumi M, Abe J, Haendeler J, Huang Q, Berk BC (2000) Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. J Biol Chem 275 (16):11706-11712. doi:10.1074/jbc.275.16.11706
36. Ushio-Fukai M, Alexander RW, Akers M, Griendling KK (1998) p38 Mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy. J Biol Chem 273 (24):15022-15029. doi:10.1074/jbc.273.24.15022
37. Schwartz RS, Henry TD (2002) Pathophysiology of coronary artery restenosis. Rev Cardiovasc Med 3 Suppl 5:S4-9
38. Kibos A, Campeanu A, Tintoiu I (2007) Pathophysiology of coronary artery in-stent restenosis. Acute Card Care 9 (2):111-119. doi:10.1080/17482940701263285
39. Kauffman RF, Bean JS, Zimmerman KM, Brown RF, Steinberg MI (1991) Losartan, a nonpeptide angiotensin II (Ang II) receptor antagonist, inhibits neointima formation following balloon injury to rat carotid arteries. Life Sci 49 (25):Pl223-228. doi:10.1016/0024-3205(91)90298-p
40. Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AH, Ishida Y, Inagami T, Saruta T (2004) Inhibition of diabetic nephropathy by a decoy peptide corresponding to the "handle" region for nonproteolytic activation of prorenin. J Clin Invest 114 (8):1128-1135. doi:10.1172/jci21398

41. Roberts MJ, Young IS, Trouton TG, Trimble ER, Khan MM, Webb SW, Wilson CM, Patterson GC, Adgey AA (1990) Transient release of lipid peroxides after coronary artery balloon angioplasty. Lancet 336 (8708):143-145. doi:10.1016/0140-6736(90)91661-s

Tables

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

Names of miRNA	Sequence (5'to 3')
RNAi-(P)RR-1F	TGCTGATAGGTTGCCCACAGCAAGCCGTTTTGGCC ACTGACTGACGGCTTGCTGGGCAACCTAT
RNAi-(P)RR-1R	CCTGATAGGTTGCCCAGCAAGCCGTCAGTCAGTGG CCAAAACGGCTTGCTGTGGGCAACCTATC
RNAi-(P)RR-2F	TGCTGTTTAGACTCTCCAAGACAAGGGTTTTGGCC ACTGACTGACCCTTGTCTGAGAGTCTAAA
RNAi-(P)RR-2R	CCTGTTTAGACTCTCAGACAAGGGTCAGTCAGTGG CCAAAACCCTTGTCTTGGAGAGTCTAAAC
RNAi-(P)RR-3F	TGCTGTTCACTGTCACCATAATGGTAGTTTTGGCCA CTGACTGACTACCATTAGTGACAGTGAA
RNAi-(P)RR-3R	CCTGTTCACTGTCACTAATGGTAGTCAGTCAGTGG CCAAAACTACCATTATGGTGACAGTGAAC
RNAi-(P)RR-4F	TGCTGTTCGGTTGCAGGTTCTCGAAAGTTTTGGCC ACTGACTGACTTTCGAGACTGCAACCGAA
RNAi-(P)RR-4R	CCTGTTCGGTTGCAGTCTCGAAAGTCAGTCAGTGG CCAAAACTTTCGAGAACCTGCAACCGAAC
RNAi-(P)RR-NC-F	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCC ACTGACTGACGTCTCCACGCAGTACATTT
RNAi-(P)RR-NC-R	CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGG CCAAAACGTCTCCACGCGCAGTACATTTC

Figures

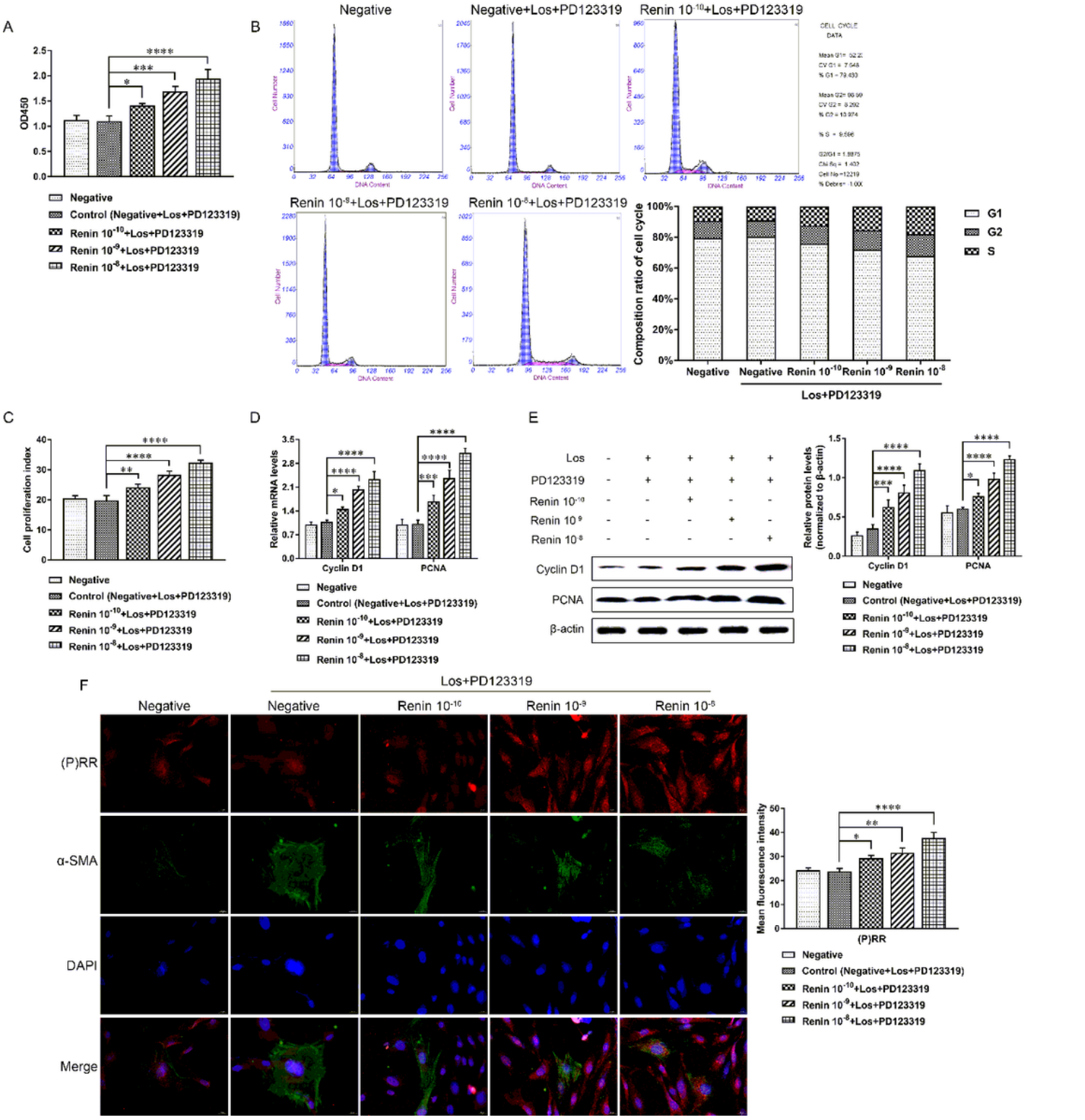

Figure 1

Renin improved proliferation of VSMCs. (A) VSMCs proliferation with different concentration of renin was performed with CCK-8 assay and presented as OD values. (B) Cell cycle of VSMCs was measure by FCM and the composition ratio of G1, G2 and S phase were shown. (C) Cell cycle of VSMCs was quantified by proliferation index. (D) Cyclin D1 and PCNA mRNA and (E) protein expressions of VSMCs treated with different concentration of renin were determined by RT-PCR and WB. (F) The expression of (P)RR was

measured by immunofluorescent staining at 400× magnification in VSMCs with renin and quantified as MFI. Scale bar=20 μm. Bars represented the mean ± S.D. from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

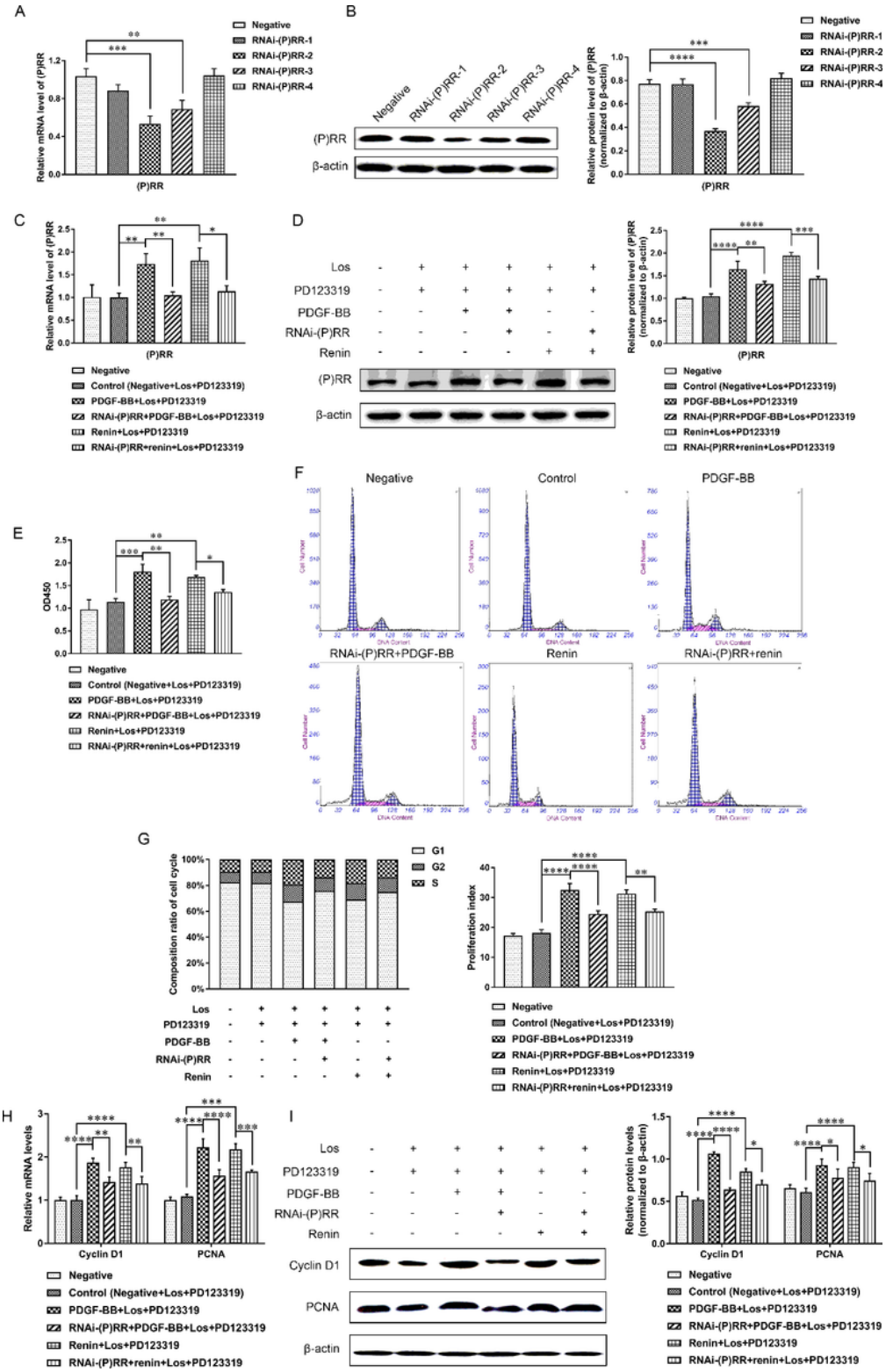

Figure 2

Silencing (P)RR restrained VSMCs proliferation. (A) The (P)RR mRNA (B) and protein levels were determined by RT-PCR and WB to choose the most effective miRNA of (P)RR silencing. (C) VSMCs were

treated with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR for measuring the mRNA or (D) protein levels by RT-PCR and WB. (E) VSMCs proliferation with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR was performed with CCK-8 assay and presented as OD values. (F) Cell cycle of VSMCs with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR was measure by FCM. (G) The composition ratio of G1, G2, S phase and proliferation index were shown. (H) Cyclin D1 and PCNA mRNA and (I) protein expressions of VSMCs treated with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR were determined by RT-PCR and WB. Bars represented the mean ± S.D. from at least three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

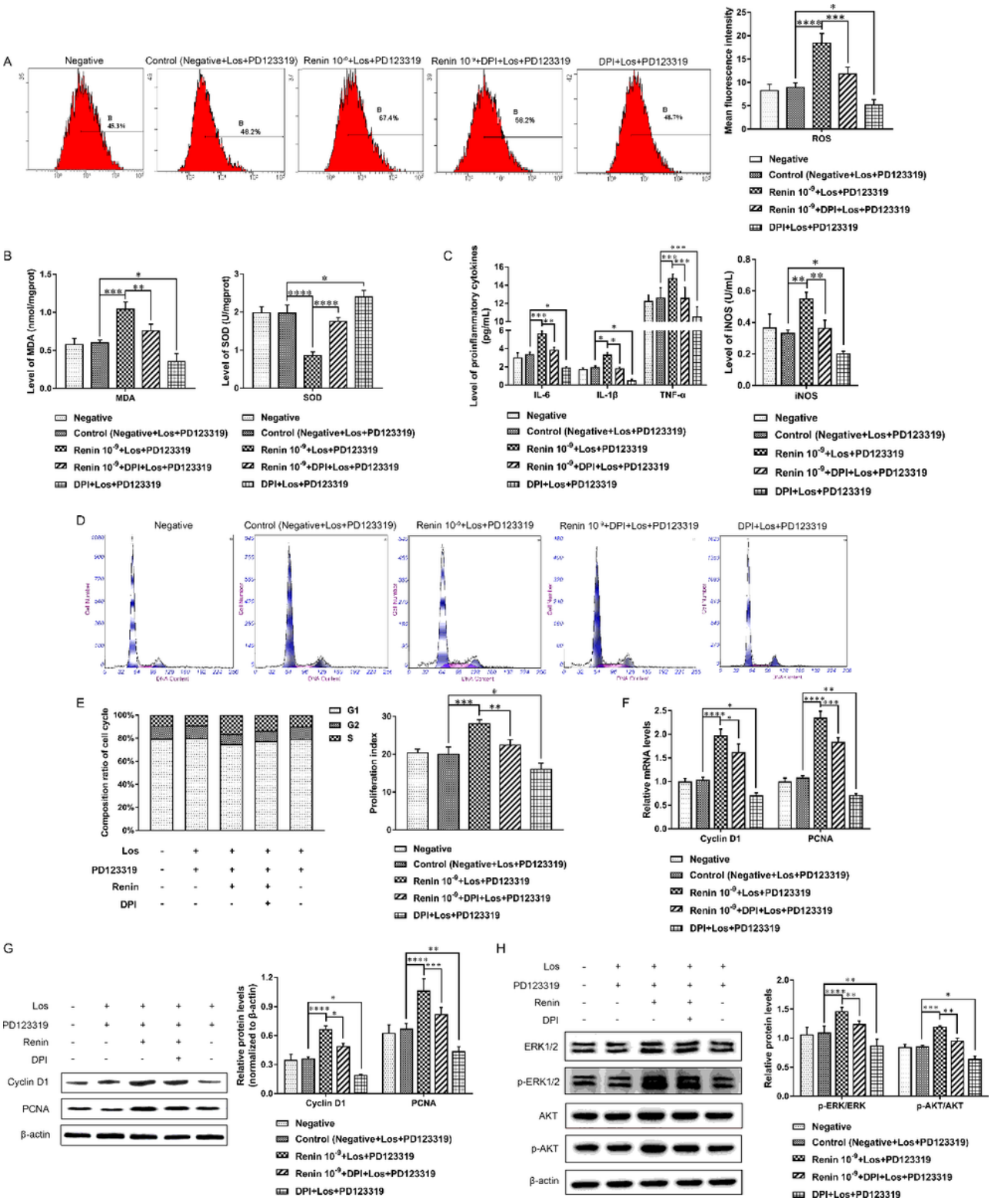

Figure 3

Renin induced VSMCs proliferation via activating oxidative stress and inflammatory response. (A) The ROS level of VSMCs treated with Los, PD123319, renin, or DPI was measured by FCM and quantified by MFI. (B) The MDA and SOD levels of VSMCs treated with Los, PD123319, renin, or DPI were measured by ELISA. (C) The IL-6, TNF-α, IL-1β, and iNOS levels of VSMCs treated with Los, PD123319, renin, or DPI were measured by ELISA. (D) Cell cycle of VSMCs with Los, PD123319, renin, or DPI was measure by

FCM. (E) The composition ratio of G1, G2, S phase and proliferation index were shown. (F) The Cyclin D1 and PCNA mRNA and (G) protein expressions of VSMCs treated with Los, PD123319, renin, or DPI were determined by RT-PCR and WB. (H) The ERK1/2, p-ERK1/2, AKT, p-AKT expressions of VSMCs treated with Los, PD123319, renin, or DPI were measured by WB and presented as the ratio of p-ERK/ERK and p-AKT/AKT. Bars represented the mean ± S.D. from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

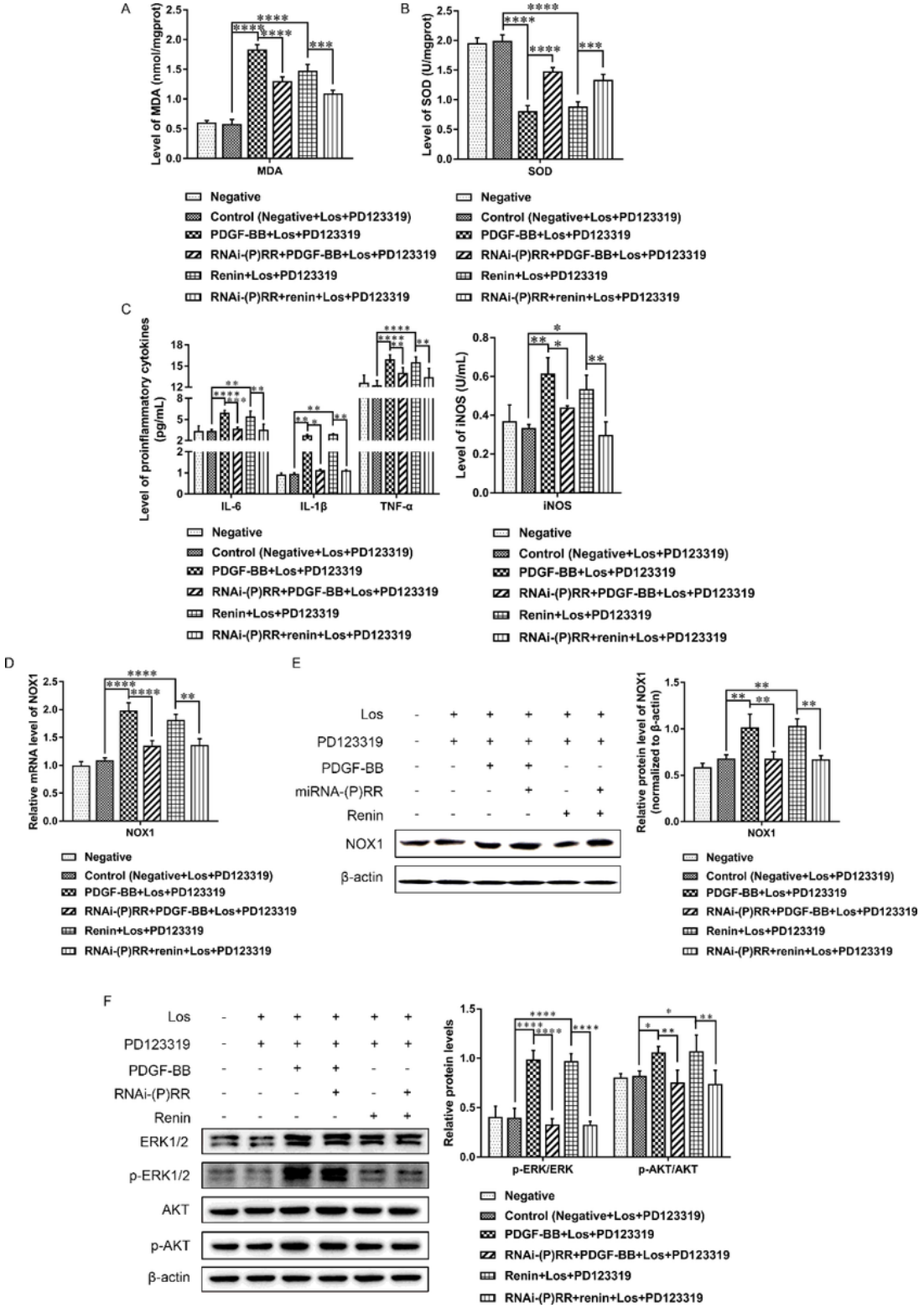

Figure 4

Silencing (P)RR decreased the levels of oxidative stress and inflammation. (A) The MDA and (B) SOD levels of VSMCs treated with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR were measured by ELISA. (C) The IL-6, TNF-α, IL-1β, and iNOS levels of VSMCs treated with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR were measured by ELISA. (D) The NOX1 mRNA and (E) protein expressions of VSMCs treated with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR were determined by RT-PCR and WB. (F) The ERK1/2, p-ERK1/2, AKT, p-AKT expressions of VSMCs treated with Los, PD123319, PDGF-BB, renin, or RNAi-(P)RR were measured by WB and presented as the ratio of p-ERK/ERK and p-AKT/AKT. Bars represented the mean ± S.D. from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

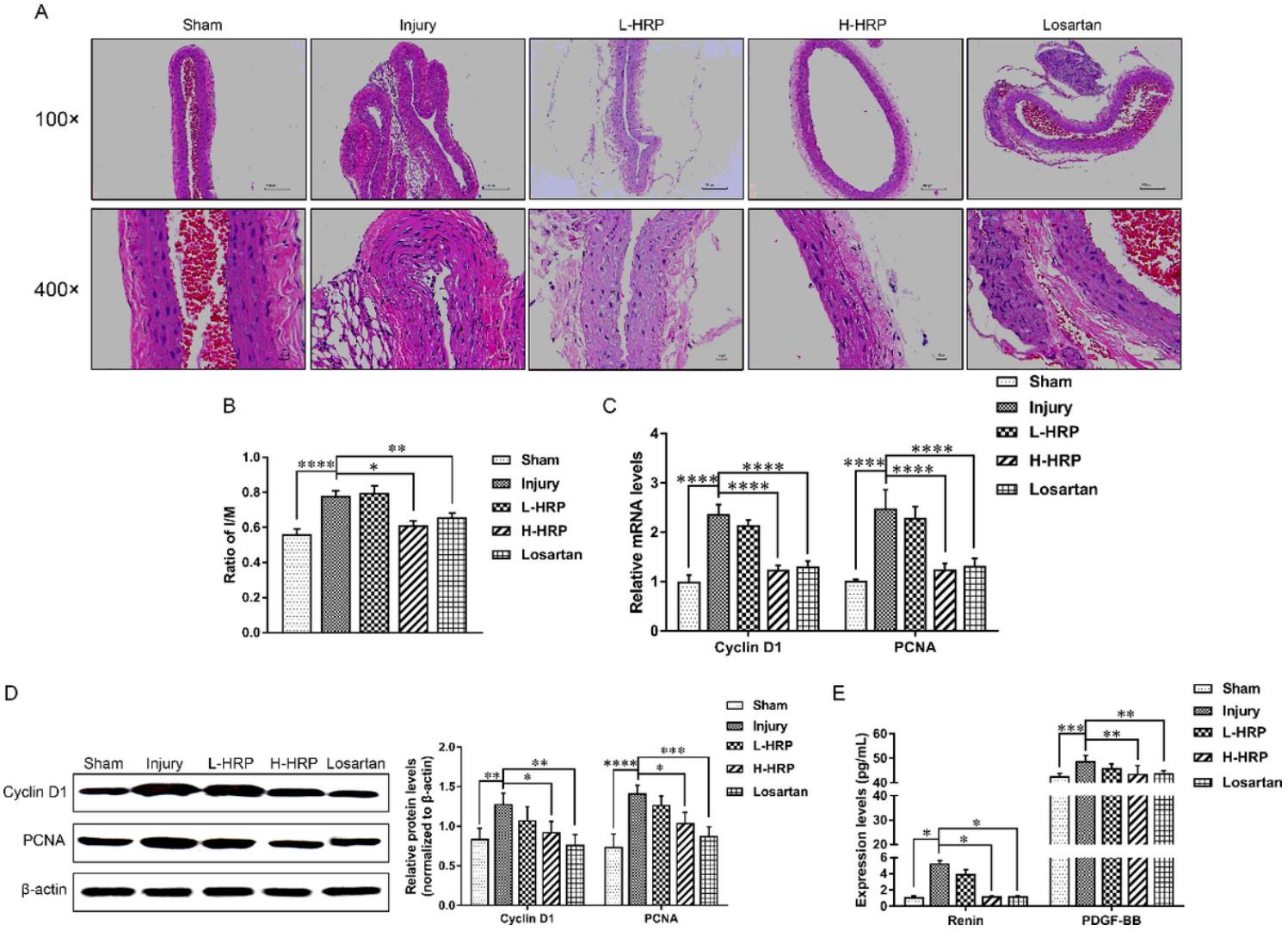

Figure 5

Inhibition of (P)RR restrained neointimal hyperplasia after common carotid artery injury of rats. (A) The neointimal hyperplasia in each group with L-HRP, H-HRP or losartan was observed by H&E staining with 100× and 400× magnification. (B) The neointimal hyperplasia was quantified by the intima/media area ratio (I/M). (C) The cyclin D1 and PCNA mRNA and (D) protein expressions of rats treated with L-HRP, H-HRP or losartan were determined by RT-PCR and WB. (E) The levels of renin and PDGF-BB were

determined by ELISA. Bars represented the mean ± S.D. from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

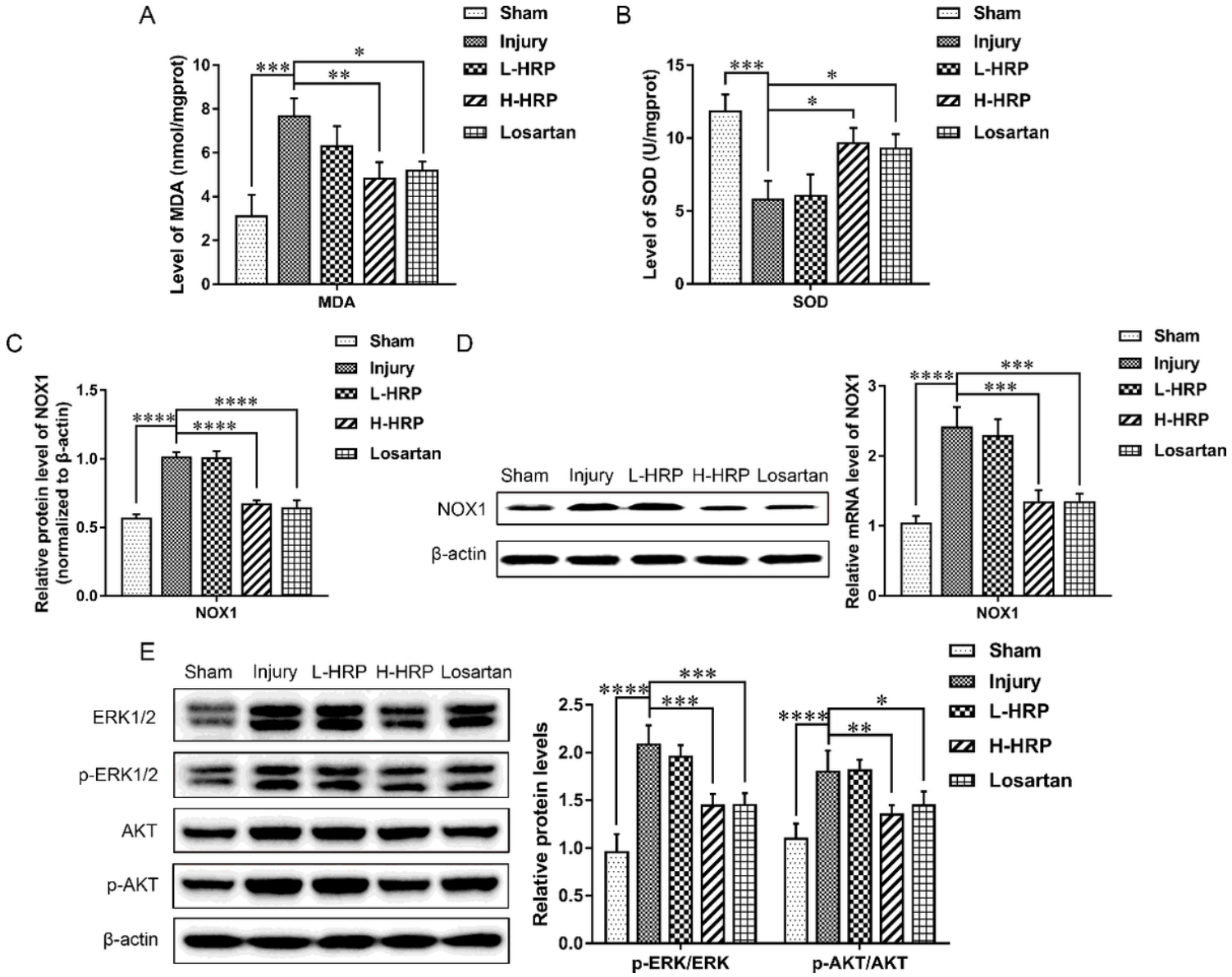

Figure 6

Inhibition of (P)RR restrained NOX1-mediated oxidative stress by down-regulating ERK1/2-AKT pathway. (A) The MDA and (B) SOD levels of rats treated with L-HRP, H-HRP or losartan were measured by ELISA. (C) The NOX1 mRNA and (D) protein expressions of rats treated with L-HRP, H-HRP or losartan were determined by RT-PCR and WB. (E) The ERK1/2, p-ERK1/2, AKT, p-AKT expressions of rats treated with L-HRP, H-HRP or losartan were measured by WB and presented as the ratio of p-ERK/ERK and p-AKT/AKT. Bars represented the mean ± S.D. from at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.