Original Paper

Sinomenine Attenuates Angiotensin II-Induced Autophagy via Inhibition of P47-Phox Translocation to the Membrane and Influences Reactive Oxygen Species Generation in Podocytes

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Key Words
Sinomenine • Podocyte • Autophagy • Angiotensin II • NADPH oxidase • p47-phox

Abstract
Background/Aims: Sinomenine, a pure alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum*, and sinomenine hydrochloride (SN) has been successfully used for the therapy of rheumatoid arthritis (RA) and kidney diseases. Autophagy is a cytoprotective mechanism used by podocytes and other cells to alleviate the effects of oxidative stress, and angiotensin II (Ang II) significantly promotes podocyte autophagy. However, excessive autophagy may lead to cell death and podocyte depletion. The present study evaluated the effect of SN in podocytes induced by Ang II. ***Methods:*** Podocytes were pretreated with graded concentrations (10^{-8} M ~ 10^{-4} M) of SN and then stimulated with Ang II. The LC3B protein and the p47-phox membrane fraction were measured by Western blot. Autolysosomes were assessed by transmission electron microscopy. FACS was used to quantify the ROS produced by podocytes. The translocation of p47-phox to the membrane was investigated by immunofluorescence. ***Results:*** The 10^{-8} M ~ 10^{-4} M of SN alone did not effect ROS generation or podocyte autophagy. The 10^{-8} M and 10^{-6} M SN attenuated Ang II-induced autophagy in podocytes. Furthermore, SN decreased the level of ROS generation in Ang II-induced podocytes via inhibition of NOX subunit p47-phox translocation to the membrane. ***Conclusion:*** The appropriate concentration of SN attenuated Ang II-induced podocyte autophagy through ROS generation, at least in part, by regulating NOX subunit p47-phox translocation to the membrane.

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159

Wang/Cai/Tang/Zhang/Gao/Xie/Mou/Wu/Wang/Zhang: SN Attenuates Ang II-Induced
Autophagy in Podocytes

Introduction

Podocytes, as highly differentiated cells, are important constituents of the glomerular filtration barrier [1], which prevent the loss of plasma proteins and have numerous critical biological functions. Podocytes are often exposed to various damaging factors, which can potentially induce oxidative stress and DNA damage [2-5]. Injured podocytes play a key factor in the pathogenesis of glomerular diseases and the progression of renal failure. Podocytes survival is dependent on autophagy to efficiently sequester unwanted or damaged proteins/organelles into autophagosomes [6]. Podocytes are cells with high levels of basal autophagy [7, 8]. Autophagy includes the autophagic flux consisting of the fusion of autophagosomes to lysosomes and the lysosomal enzymatic degradation of these autophagic substances, which keeping podocytes in a differentiated and functional status [9]. However, excessive autophagy may lead to cell death [10].

Autophagy is a response to diverse conditions of stress [11, 12], and it has a dual role in cell survival and cell death. Autophagic cell death has been observed in response to oxidative stress [13]. Increasing evidence argues for oxidative stress acting as the converging point of these stimuli, with reactive oxygen species (ROS) initiating autophagosome formation and modulating the autophagy process [14, 15]. The concentration of endogenous angiotensin II (Ang II) in the kidney cortical and medullary regions were $60\sim100$ times higher than in the arterial plasma [16]. Ang II also promotes podocyte autophagy that is dependent on the ROS generation [11].

There are two major sources for ROS production in cells, mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX), which actively produces superoxide across the membranes [17, 18]. NOX is a multi-component enzyme for ROS generation, consisting of a catalytic subunit NOX2/gp91-phox and p22 phox, and several cytosolic regulatory subunits, including p47-phox and p67-phox [19, 20]. The p47-phox protein, a major regulatory subunit, is located at the plasma membrane and assembles with other subunits that are required for the full activation of NOX [21].

Sinomenine ($9\alpha,13\alpha,14\alpha$)-7,8-didehydro-4-hydroxy-3,7-dimethoxy-17-methyl-morphinane-6-one], a pure alkaloid extracted from the Chinese medicinal plant *Sinomenium acutum*, possesses various pharmacological activities due to its complex chemical composition, and its pharmacological profile includes immunosuppression, arthritis amelioration, and anti-inflammation. Sinomenine hydrochloride (SN) (Fig. 1, from the datasheet of Sinomenine hydrochloride produced by abcam, ab141190) has successfully been used in the therapy of rheumatoid arthritis (RA) and kidney diseases. It has superior therapeutic effects and relatively few side effects [22-24], but the mechanism by which SN acts remains unclear. Recent studies indicated that the lower concentrations ($10^{-14} \sim 10^{-4}$ M) of SN can decrease microglial cell ROS generation and confers neuroprotection [25, 26]. Whereas, the higher concentrations ($10^{-4} \sim 10^{-1}$ M) of SN also have anti-inflammatory effects via the regulation of autophagy activities [27, 28].

On the basis of ROS as an important mediator in autophagy, we hypothesized the lower concentrations ($10^{-8} \sim 10^{-4}$ M) of SN may attenuate podocyte autophagy. In this study, we investigated the effects of SN on Ang II-induced podocyte autophagy and the mechanism of protection caused by its regulation of the NOX subunit, p47-phox.
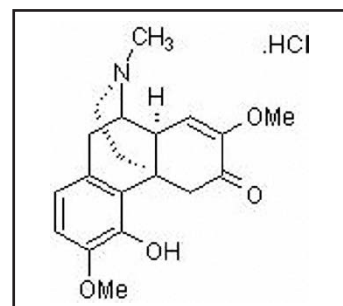
Fig. 1. The structure of sinomenine hydrochloride (SN) .

Materials and Methods

Reagents

Fetal bovine serum (FBS) and RPMI 1640 were purchased from Gibco (NY, USA). The ITS (containing 10 μg/ml insulin, 0.55 mg/ml human transferrin and 0.5 μg/ml sodium selenite), rabbit anti-human LC3B polyclonal antibody and diphenyleneiodonium chloride (DPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Angiotensin II (Ang II) was purchased from Biovision (Milpitas, USA). Sinomenine hydrochloride (SN) was obtained from Zhengqing Pharmaceutical Group (HuNan, China). Rabbit anti-human p47-phox polyclonal antibody for immunofluorescence was purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit anti-human p47-phox polyclonal antibody for Western blot was purchased from Bioworld (MN, USA). The Membrane Protein Extraction kit was purchased from BestBio (ShangHai, China). The Enhanced BCA Protein Assay Kit, reactive oxygen species kit, mouse anti human β-actin monoclonal antibody, HRP-labeled goat anti-rabbit IgG, HRP-labeled goat anti-mouse IgG, Alexa Fluor 488-labeled goat anti-rabbit IgG, Hoechst 33342, RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) and the protease inhibitor phenylmethanesulfonylfluoride (PMSF) were all purchased from Beyotime (Shanghai, China).

Podocyte culture and treatment

Immortalized human podocytes (AB8/13) were a kind gift from Dr. Moin A. Saleem (University of Bristol, UK). Podocytes were maintained in RPMI 1640 containing 10% FBS, ITS, penicillin and streptomycin at 33°C in 95% air, 5% CO_2. After reaching 70% confluence, the cells were transferred to 37°C in 95% air, 5% CO_2 and cultured for 14 days for differentiation [29]. The differentiated podocytes show morphology with multiple foot processes, and only differentiated cells were used for experiments. Cells were stimulated with 10^{-7} M Ang II for 24 h as previously described [11] or with medium alone. In some experiments, cells were treated with graded concentrations (10^{-8} M ~ 10^{-4} M) of SN alone for 24 h or pretreated for 1 h before stimulation with Ang II.

Reactive oxygen species determination

The level of intracellular ROS produced in podocytes was quantified using the fluorescent signal from the redox-sensitive fluoroprobe 2', 7'- dichlorofluorescein diacetate (DCFH-DA) according to the protocol provided by the manufacturer (Beyotime, China). Cells were grown in 6-well plates in serum-free culture medium for 24 h. The podocytes were treated as previously described. The cells were then washed twice with PBS and then incubated with DCFH-DA (5 μM) in medium at 37°C for 30 min in the dark. Then, the cells were washed with PBS and the fluorescent intensity was measured with a flow cytometer using an excitation wavelength of 488 nm and an emission wavelength of 525 nm (BD Accuri C6, USA).

Immunofluorescence analysis

Podocytes were plated on poly-L-lysine treated coverslips at 37°C for 24 h. After treatment, the cells were fixed with 4% paraformaldehyde for 15 min and blocked with 5% bovine serum albumin (BSA) for 1 h. Then, the cells were incubated with rabbit anti human p47-phox polyclonal antibody (diluted 1:50, Santa Cruz) at 4°C overnight and then conjugated with Alexa Fluor 488-labeled goat anti-rabbit IgG (diluted 1:1000) at room temperature for 1 h in the dark. After staining with Hoechst 33342 for 5 min, the coverslips were sealed with glycerol jelly mounting medium for detection. Images were captured with a fluorescence microscope (Nikon, Japan) from random microscope fields and analyzed using Image-Pro Plus 6.

Protein Extraction and Western blot analysis

The total LC3B protein and membrane protein p47-phox of the podocytes were analyzed by Western blot. During the total protein extraction, the podocytes were washed with EDTA and harvested using a cell scraper after treatment. Next, the samples were lysed with RIPA lysis buffer containing PMSF. For the membrane protein extraction, each sample contained 5×10^6 podocytes, which were cultured in 75 cm² plastic culture flasks. After treatment, cells were washed with EDTA and collected with a cell scraper. Then, all procedures were performed according to the protocol for the Membrane Protein Extraction kit for extracting the membrane protein p47-phox (BestBio, China). Protein concentrations of the samples were determined using a BCA protein assay. Each protein lysate was separated by 10% SDS-PAGE and transferred

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to polyvinylidene difluoride (PVDF) membranes. Subsequently, PVDF membranes were blocked with 5% nonfat milk at room temperature for 1 h and then incubated with the appropriate dilution of primary antibodies (p47-phox, diluted 1:500; LC3B, diluted 1:1000; β-actin diluted 1:500) at 4°C overnight. Then, the membranes were incubated for 1 h at room temperature with HRP-labeled goat anti-rabbit IgG or HRP-labeled goat anti-mouse IgG secondary antibody (diluted 1:1000). Following washing, the membranes were detected by ECL according to the manufacturer's instructions.

Transmission electron microscopy investigation

Podocytes were fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide. Then, the samples were dehydrated in a graded ethanol series and propylene oxide and embedded on molds containing pure Spurr's resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and imaged with a JEM-1400 plus electron microscope (JEOL, Japan).

Statistical analysis

All data are expressed as the means ± SEM, and one-way ANOVA combined with Student-Newman-Keuls were used to compare the differences between multiple groups using the Graphpad Prism 6 software. A value of $p < 0.05$ was considered statistically significant.
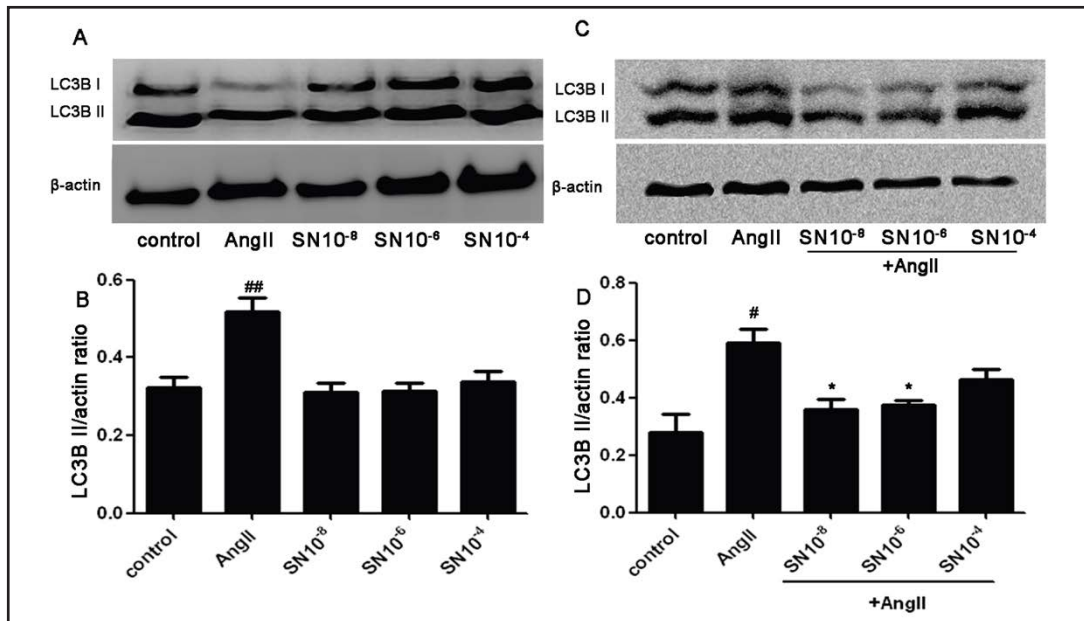
Results

Sinomenine attenuates Ang II-induced autophagy in podocytes

To study the effect of SN on Ang II-induced autophagy, we first determined whether SN alone promoted podocyte autophagy. Podocytes were incubated with 10^{-7} M Ang II or 10^{-8} M ~ 10^{-4} M of SN alone for 24 h. Total protein of the podocytes was extracted and measured by Western blot. The LC3B-II/actin ratio was used as an autophagy biomarker. As previously reported [11], the LC3B-II/actin ratio was significantly increased in Ang II-induced cells compared with the control group, but 10^{-8} M ~ 10^{-4} M SN alone had no significant effect on podocyte autophagy (Fig. 2A and B). We then determined the effect of SN on Ang II-induced podocyte autophagy. Cells were pretreated with different concentrations of SN for 1 h and then stimulated with 10^{-7} M Ang II for 24 h. The podocyte autophagy increase caused by Ang II was blocked by 10^{-8} M and 10^{-6} M SN but was not blocked in the 10^{-4} M SN pretreated group (Fig. 2C and D). This result demonstrated that the appropriate concentration of SN has a protective effect on Ang II-induced autophagy in podocytes.

Transmission electron microscopy is a direct and reliable method to observe autophagy by investigating autolysosomes formation [30]. Podocytes were incubated with 10^{-7} M Ang II after pretreatment with 10^{-8} M ~ 10^{-4} M SN. Normal podocytes showed the formation of few autophagosomes, whereas with Ang II stimulation, the number of autolysosomes increased significantly. This phenomenon was inhibited by 10^{-8} M and 10^{-6} M SN but not 10^{-4} M SN (Fig. 3A). We calculated the number of autophagosomes in five random fields from different podocytes (Fig. 3B). The results were consistent with the LC3B protein analyzed by Western blot.
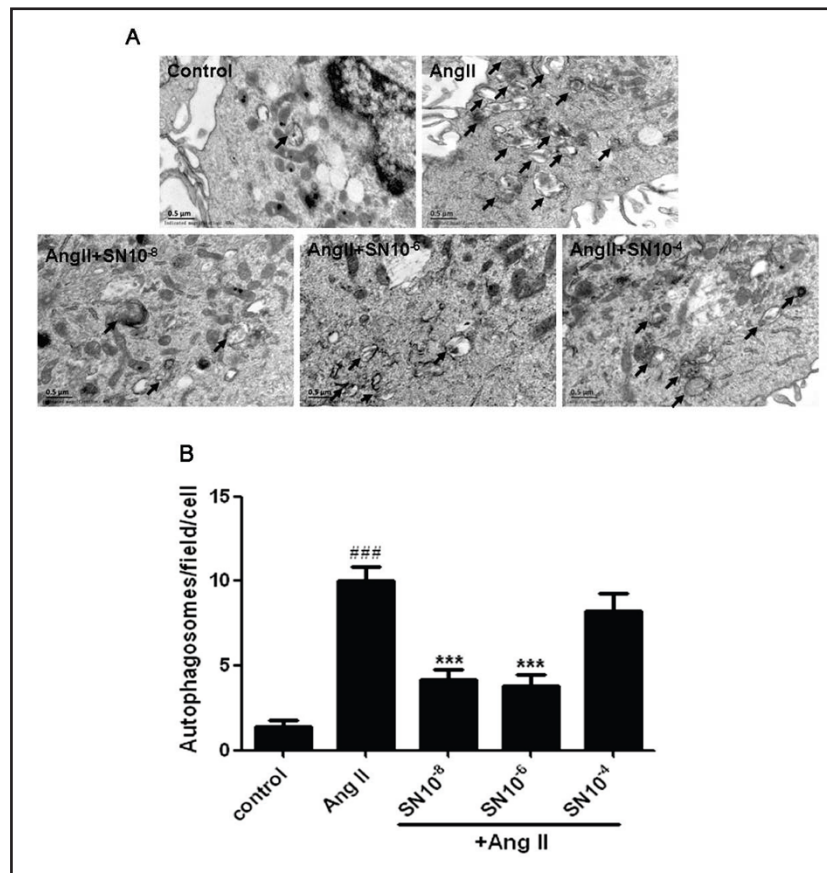
Sinomenine decreases the level of ROS generation in Ang II-induced podocytes
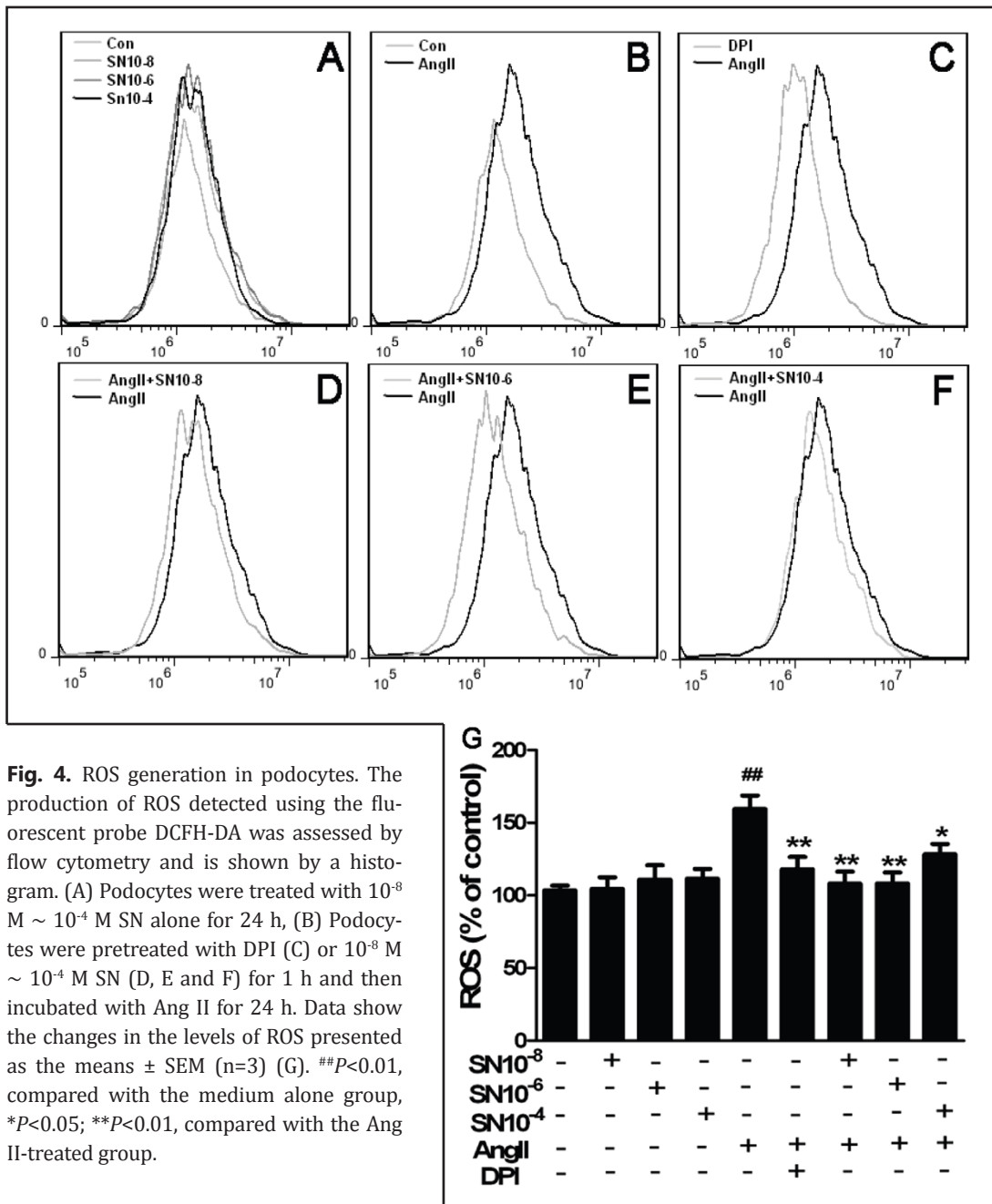
ROS has a vital role in the generation of autophagy and is significantly increased by Ang II based on previous studies [11, 17]; therefore, we further investigated the effect of SN on ROS generation on Ang II-induced podocytes. Podocytes were treated with 10^{-8} M ~ 10^{-4} M SN alone for 24 h. In some experiments, podocytes were pretreated with SN or 10 μM DPI, an inhibitor of NADPH oxidase for 1 h. Then, the pretreated podocytes were incubated with 10^{-7} M Ang II for 24 h. We found that SN alone did not affect ROS generation in podocytes (Fig. 4A). Ang II significantly promoted ROS generation compared with the medium alone group (Fig. 4B). This effect was significantly inhibited by DPI (Fig. 4C) and in the 10^{-8} M and 10^{-6} M SN pretreated groups (Fig. 4D and 4E). The 10^{-4} M SN group also showed this effect but was weaker than the lower concentration group (Fig. 4F). These results showed that the lower

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162

Wang/Cai/Tang/Zhang/Gao/Xie/Mou/Wu/Wang/Zhang: SN Attenuates Ang II-Induced
Autophagy in Podocytes

Fig. 2. The expression of LC3B in podocytes. The LC3BII/actin ratio is related to autophagy of podocytes. (A and B) Podocytes were treated with SN (10^{-8} M ~ 10^{-4} M) alone for 24 h. (C and D) Podocytes were pretreated with SN (10^{-8} M ~ 10^{-4} M) for 1 h and then treated with Ang II for 24 h. Data are presented as the means ± SEM (n=3). $^{\#}P<0.05$; $^{\#\#}P<0.01$, compared with medium alone; $^{*}P<0.05$, compared with the Ang II-treated group.

Fig. 3. Autolysosome formation was detected by transmission electron microscopy (TEM). (A) The representative autolysosomes are indicated by black arrows in the medium group. Ang II-stimulated groups with or without pretreatment using 10^{-8} M ~ 10^{-4} M SN. (B) Statistical data are shown as the numbers of autophagosomes counted in random fields from different podocytes. Data are presented as the means ± SEM (n=5, from five different cells). $^{\#\#\#}P<0.001$, compared with the medium alone group; $^{***}P<0.001$, compared with the Ang II-treated group.

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163

Wang/Cai/Tang/Zhang/Gao/Xie/Mou/Wu/Wang/Zhang: SN Attenuates Ang II-Induced
Autophagy in Podocytes

Fig. 4. ROS generation in podocytes. The production of ROS detected using the fluorescent probe DCFH-DA was assessed by flow cytometry and is shown by a histogram. (A) Podocytes were treated with 10^{-8} M $\sim 10^{-4}$ M SN alone for 24 h, (B) Podocytes were pretreated with DPI (C) or 10^{-8} M $\sim 10^{-4}$ M SN (D, E and F) for 1 h and then incubated with Ang II for 24 h. Data show the changes in the levels of ROS presented as the means $\pm$ SEM (n=3) (G). $^{\#\#}P<0.01$, compared with the medium alone group, $^*P<0.05$; $^{**}P<0.01$, compared with the Ang II-treated group.

concentrations (10^{-8} M and 10^{-6} M) of SN decreased the ROS generation induced by Ang II better than the higher concentration (10^{-4} M) of SN (Fig. 4G), indicating that the appropriate concentrations of SN in podocytes protects against excessive ROS generation.

*Sinomenine prevents p47-phox translocation to the membrane*

The translocation to the membrane of p47-phox, the main regulatory subunit of NOX, plays an important role in the full activation of NOX and ROS generation [21]. A previous study found that p47-phox translocation from the cytosol to membrane is induced by Ang II [31]. Here, we determined whether p47-phox translocation was prevented by SN in podocytes. Using fluorescence microscopy, we observed p47-phox expression on the membrane of podocytes induced by 10^{-7} M Ang II for 24 h, as shown in (Fig. 5B). The p47phox content on

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Fig. 5. P47-phox localization on the membrane was detected by immunofluorescence staining. P47-phox expressed under the basal state (A), treated with 10^{-7} M Ang II (B) or pretreated with 10^{-8} M SN (C), 10^{-6} M SN (D), and 10^{-4} M SN (E) before treatment with 10^{-7} M Ang II (original magnification of 400x). Data are presented as the mean fluorescence intensity (MFI) as the means ± SEM (n=3, from three random fields of each sample) (F). $^{###}P<0.001$, compared with the basal state (control), *** $P<0.001$, compared with the Ang II group.

the membrane was greater for the treated cells than the control (Fig. 5A). However, podocytes pretreated with 10^{-8} M and 10^{-6} M SN for 1 h showed sporadic p47-phox membrane staining, suggesting that SN prevented Ang II-induced p47-phox translocation (Fig. 5C and D). However, 10^{-4} M SN showed weaker effects compared with the lower concentrations of SN. (Fig. 5E). The mean fluorescence intensity (MFI) was analyzed and is shown in (Fig. 5F).

Consistent with the results of the fluorescence microscopy study, we also analyzed the expression of p47-phox from the membrane fraction of podocytes using Western blot (Fig. 6A). The increase of p47-phox in the membrane fraction of podocytes after Ang II treatment was blocked in the presence of SN at 10^{-8} M and 10^{-6} M, but 10^{-4} M SN showed only a partial preventative effect (Fig. 6B). These results demonstrate that SN is protective at low concentrations, but the effect is impaired at a higher concentration.
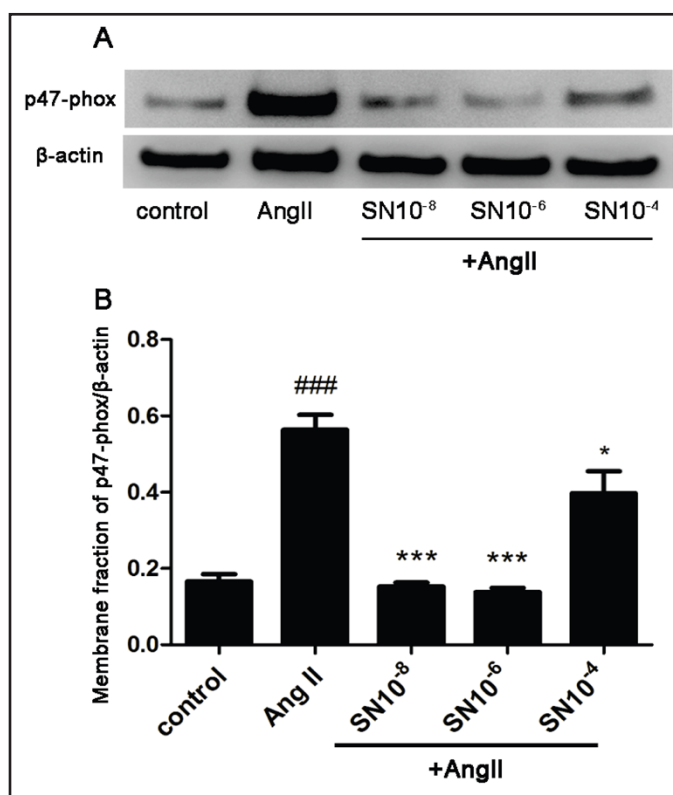
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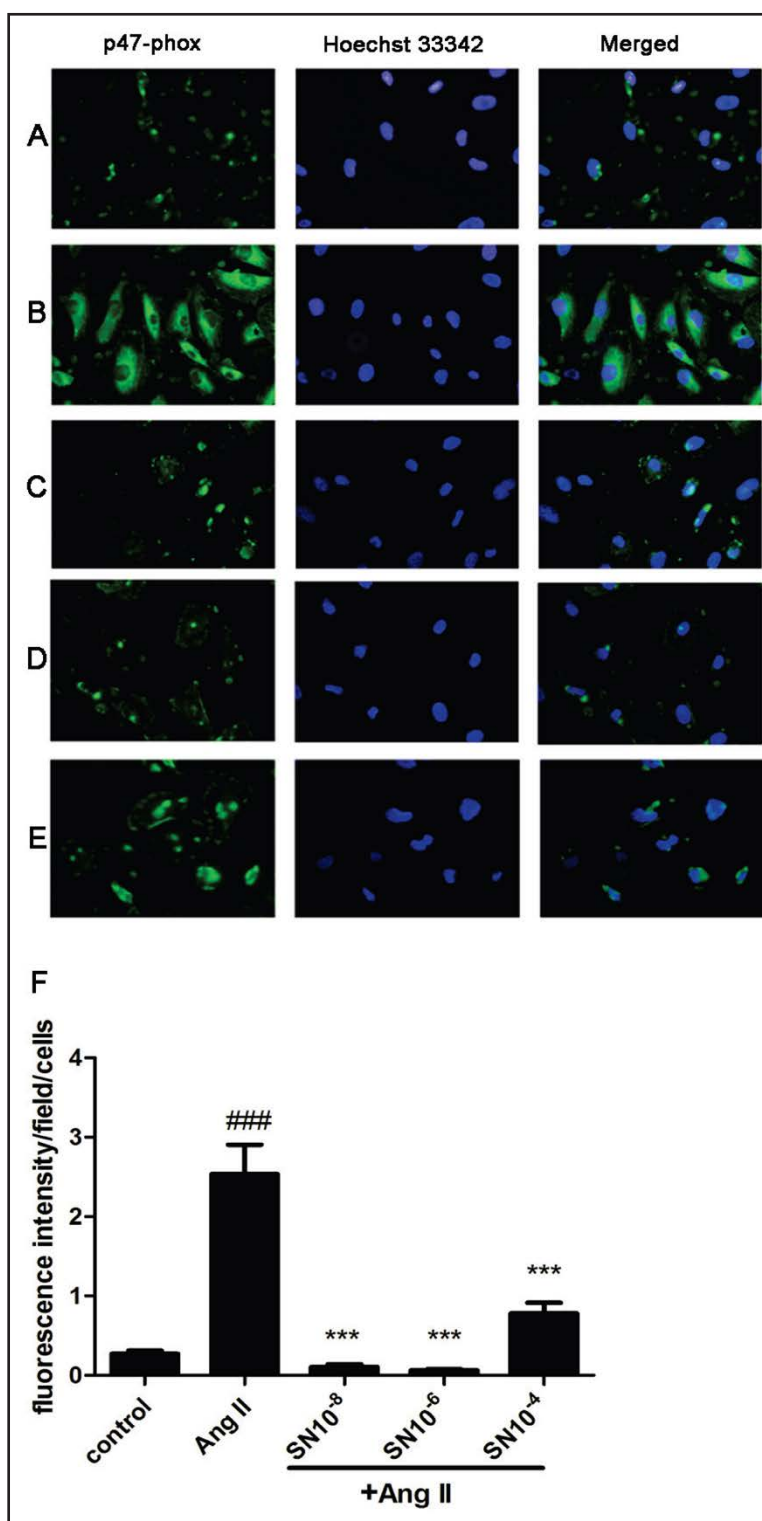
Discussion

Autophagy, which literally means "self-eating", is a catabolic process aimed at recycling cellular components and damaged proteins or organelles in response to diverse conditions of stress, such as oxidative stress [32]. Basal autophagy plays an essential role in the maintenance of cellular homeostasis, whereas excessive autophagy may lead to cell death [10, 33]. Accumulating evidence showed that ROS plays an essential role for autophagy regulation [11, 12, 32, 34, 35]. Ang II stimulates vascular smooth muscle cell ROS derived from NOX [35] and promotes autophagy in podocytes through ROS [11]. We also found that Ang II significantly increased the ROS and autophagy levels in podocytes, and the effect was inhibited with 10^{-8} M and 10^{-6} M SN. Interestingly, the high concentration of 10^{-4} M SN failed to show a protective effect, indicating that SN has a protective effect dependent on the concentration. Some reports indicated that SN itself increased cell autophagy [27, 28]. However, it is notable that

Fig. 6. The expression on p47-phox in the membrane fraction was assessed by Western blotting. Changes in the level of p47-phox expression in the membrane fraction from podocytes incubated in medium alone or pretreated with different concentration of SN (10^{-8} M $\sim$ 10^{-4} M) and then stimulated with 10^{-7} M Ang II (A). Data are presented as the p47-phox/β-actin ratio as the means $\pm$ SEM (n=3) (B). ###$P<0.001$, compared with the medium group; ***$P<0.001$, *$P<0.05$, compared with the Ang II group.

the concentrations of SN in those studies ranged from 10^{-4} M $\sim$ 10^{-1} M, which was higher than the concentration used in this study. In another experiment, SN inhibited LPS-induced ROS increase at concentrations of 10^{-14} M and 10^{-5} M but not 10^{-10} M [25]. This demonstrated that different concentrations of SN have distinct effects on cell autophagy.

Not only do the concentrations but also the length of stimulation affects the SN protective activity. An experiment with BV2 microglial cells induced with oligomeric amyloid-β (ADDL) showed that pretreatment with SN for 1.5 h successfully inhibited ADDL-induced ROS increase, whereas when ADDL and SN were added at the same time, SN failed to show the effect [26]. The study also found that SN does not affect the basal level of ROS in cells, which we also found in our study. These data suggest that multiple signaling pathways are involved in the SN inhibition mechanism, and different concentrations of SN may have different effects on cells.

In this study, we selected concentrations of SN from 10^{-8} M to 10^{-4} M, and we found that SN significantly inhibited p47-phox expression on the membrane of Ang II-induced podocytes. This subsequently leads to decreased NOX-mediated ROS generation. However, there are some limitations to using laser confocal microscopy for investigating p47-phox translocation from the cytoplasm to the membrane in our study. The p47-phox protein contains two SH3 domains for binding to p22-phox and a polybasic auto-inhibitory region. These domains are

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166

Wang/Cai/Tang/Zhang/Gao/Xie/Mou/Wu/Wang/Zhang: SN Attenuates Ang II-Induced
Autophagy in Podocytes

masked in the resting state. Phosphorylation of p47-phox exposes these two SH3 domains for p22-phox binding and activates NOX [19, 20]. We propose that SN shows its protective effect by interacting with p47-phox and its domains, although this requires further studies for confirmation.

Conclusions

Our study demonstrated that the protective effect of SN attenuates Ang II-induced podocyte autophagy and that SN-mediated ROS generation decreased in part via regulation of the NOX subunit p47-phox translocation to the membrane.

Disclosure Statement

All authors declare that they have no competing interests.

Acknowledgments

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