Life Sciences 216 (2019) 140–146

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Genistein inhibits Ang II-induced CRP and MMP-9 generations via the ER-p38/ERK1/2-PPARγ-NF-κB signaling pathway in rat vascular smooth muscle cells

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ARTICLE INFO

Keywords:
Genistein
C-reactive protein
Matrix metalloproteinase 9
Angiotensin II
Vascular smooth muscle cells
Atherosclerosis
Inflammation

ABSTRACT

Aims: C-reactive protein (CRP) and matrix metalloproteinase (MMP)-9 are involved in the inflammation of atherosclerosis lesions. Genistein (Gen) has been demonstrated to exert beneficial effect on the cardiovascular system. However, it remains unclear whether Gen produces anti-inflammatory effect in vascular smooth muscle cells (VSMCs). Therefore, we investigated the effects of Gen on CRP and MMP-9 expressions induced by angiotensin (Ang) II in VSMCs and the related molecular mechanism.

Main methods: Rat VSMCs were cultured, and Ang II was used as a stimulant for CRP and MMP-9 expressions. CRP level was measured by ELISA. The mRNA and protein expressions of related indexes were identified by reverse transcription-polymerase chain reaction and western blot, respectively.

Key findings: Gen inhibited Ang II-stimulated CRP and MMP-9 mRNA and protein expressions in concentration- and time-dependent manners. Additionally, Gen ameliorated Ang II-induced p-ERK1/2, p-p38 and NF-κB expressions, antagonized Ang II-downregulated peroxisome proliferation-activated receptor (PPAR) γ and estrogen receptor (ER) β expressions. After treating the VSMCs with GW9662 or ICI182780 in Gen treated groups, inhibitory effect of Gen on CRP and MMP-9 expressions were antagonized in Ang II-stimulated VSMCs. The treatment of VSMCs with ICI182780 abolished downregulations of p-p38/p-ERK1/2, and antagonized upregulation of PPARγ by Gen in Ang II-stimulated VSMCs. Moreover, the inhibitory effect of Gen on Ang II-stimulated NF-κB expression was abolished after preincubation of VSMCs with GW9662 in Gen treated groups.

Significance: Gen exerts anti-inflammatory property via the ER-p38/ERK1/2-PPARγ-NF-κB-CRP/MMP-9 signal pathway in Ang II-stimulated VSMCs.

1. Introduction

Atherosclerosis as the primary cause of cardiovascular and cerebrovascular diseases is closely correlated with systemic inflammation [1]. C-reactive protein (CRP), a biomarker of chronic inflammation, not only plays a predictive role in acute cardiovascular diseases [2], but also is involved in the development of atherosclerosis [3]. In addition to the liver, CRP is also produced in vascular smooth muscle cells (VSMCs) of atherosclerotic lesions [4].

Matrix metallopeptidase (MMP)-9 is a subtype of MMPs which is involved in tissue remodeling and migration of VSMCs from media to intima via the degradation of the extracellular matrix (ECM) and contributes to intimal thickening in atherosclerosis [5]. Serum MMP-9 levels are consistently associated with atherosclerosis and lesion vulnerability. It is observed more in unstable than in stable atherosclerotic plaques [6]. Furthermore, MMP-9 in VSMCs is linked to atherosclerotic plaques by exacerbating atherosclerotic development.

Molecular epidemiological studies demonstrate a strong inverse relationship between the consumption of soy-based diets and the risk of atherosclerosis [7]. Genistein (Gen) is a dietary-derived flavonoid abundantly present in soybeans and is known to possess various biological effects on cardiovascular protection, including estrogenic effects

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https://doi.org/10.1016/j.lfs.2018.11.036
Received 13 August 2018; Received in revised form 15 November 2018; Accepted 15 November 2018
Available online 16 November 2018

and antioxidant effects [8,9]. Chun et al. report [10] that total flavonoid intakes are inversely associated with serum CRP concentrations. Additionally, it demonstrated that Gen inhibits MMP-9 generation in patients with chronic obstructive pulmonary disease [11] and in tumor cells [12]. However, the effects of Gen on CRP and MMP-9 expressions in VSMCs have not been reported. The previous studies illustrate that angiotensin (Ang) II stimulates CRP and MMP-9 expressions in VSMCs [13,14], which are major constituents present within the media of vessels. In the present study, we aimed to explore the effects of Gen on Ang II-stimulated CRP and MMP-9 expressions in VSMCs and molecular mechanism underlying the effect.

2. Materials and methods

2.1. Materials

Gen, Ang II, SB203580, PD98059, GW9662, and pyrrolidinedithiocarbamic acid (PDTC) were provided by Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (UT, USA). The estrogen receptor (ER) β, the peroxisome proliferator-activated receptor (PPAR) γ, the nuclear factor (NF)-κB and Lamin B1 polyclonal antibodies were supplied by Proteintech (Wuhan, China). The β-actin antibody was obtained from Santa Cruz Biotechnology (CA, USA). The phospho-extracellular regulated protein kinase (p-ERK)1/2, the phospho-p38, ERK1/2 and p38 antibodies were produced by Beyotime (Jiangsu, China).

2.2. Cell culture

Male Sprague-Dawley rats (Experimental Animal Center of Xi'an Jiaotong University School of Medicine) were anesthetized by pentobarbital (50 mg/kg). VSMCs were isolated from the thoracic aorta. The cells were processed using a 1-mm chop setting in a 10-cm dish and were cultured in DMEM with 50% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Aortic VSMCs were maintained in DMEM with 10% FBS. VSMCs from passages 5 to 8 at 70–90% confluence in 6-well plates were utilized in the experiments. All the experimental procedures were performed in accordance with the international, national, and institutional rules and were approved by the Institutional Animal Care Committee of Xi'an Jiaotong University.

2.3. Cell treatment

The growth of VSMCs was arrested by incubation in 0.1% FBS DMEM for 24 h prior to use. VSMCs were pretreated with Gen at the different concentrations (10^{-4}, 10^{-5}, and 10^{-6} M) for the different durations (6, 12, 24 and 48 h) followed by the presence or absence of Ang II (10^{-7} M) for 24 h. VSMCs were preincubated with the blockers for 60 min before treatment with Gen (10^{-5} M) for 24 h and subsequently stimulated with Ang II (10^{-7} M) for another 24 h.

2.4. ELISA

CRP concentration in the supernatant of VSMCs was measured with a rat CRP ELISA kit (Westtang, Shanghai, China) following the manufacturer's protocols. The color intensity was measured at 450 nm. The sample values were then read off using the standard curve.

2.5. Western blot analysis

After the treatment, VSMCs were washed three times with ice-cold phosphate buffered saline (PBS). Whole cell lysates were prepared in 200 μL of ice-cold RIPA lysis buffer supplemented with the protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min and were

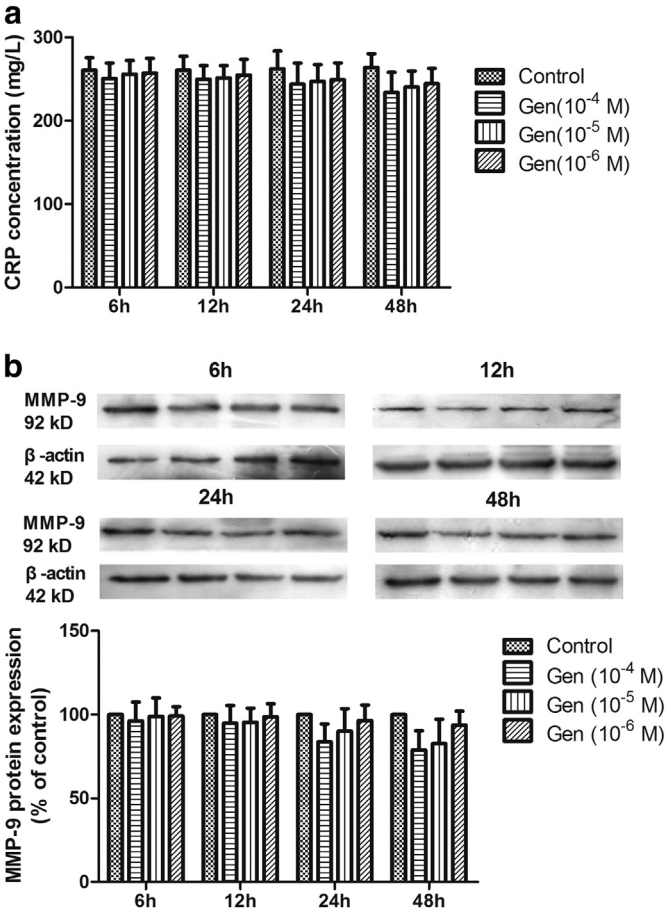

Fig. 1. Effects of genistein on protein expressions of CRP and MMP-9 in the non-stimulated VSMCs. After VSMCs were treated with or without genistein (Gen, 10^{-4}, 10^{-5}, 10^{-6} M) for 6, 12, 24 and 48 h, CRP concentration (a) and MMP-9 protein expression (b) were measured by ELISA and western blot respectively. The results from three independent experiments are expressed as the mean ± S.E.M.

then centrifuged at 4 °C for 10 min at 20,000*g*. The protein concentration was quantified with the BCA protein assay reagent kit (Thermo, USA). The samples were heated in Laemmli sample buffer (Bio-Rad) at 100 °C for 5 min. The protein extract (30 μg) was run out using a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to nitrocellulose membranes (Millipore, USA). The membranes were blocked in 10% skimmed milk in tris buffered saline buffer. After blocked for 1 h, the membranes were incubated with the primary antibodies, including anti-ERβ (1:500), anti-PPARγ (1:400), anti-Lamin B1 (1:400), anti-NF-κB (1:400), anti-p38 (1:400), anti-p-p38 (1:400), anti-ERK (1:400), anti-p-ERK (1:400) and anti-β-actin (1:1000) antibodies overnight at 4 °C in tris buffered saline tween (TBST). The membranes were washed with TBST (for 20 min, 3 times) and were then incubated with an HRP-conjugated secondary antibody (1:2000, Santa Cruz Biotechnology, CA, USA) at 37 °C for 60 min. The bands were visualized by the chemiluminescence method. After washed three times in washing buffer, the protein bands on the membrane were visualized with an ECL detection kit (Pierce, Rockford, USA).

2.6. RT-PCR

Total RNA was extracted from VSMCs with an RNAfast200 purification kit (Fastagen, Shanghai, China) following the manufacturer's instructions. cDNA was synthesized using oligo $(dT)_{18}$ Primer and RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) by the reverse transcription of mRNA. Sequence-specific PCR

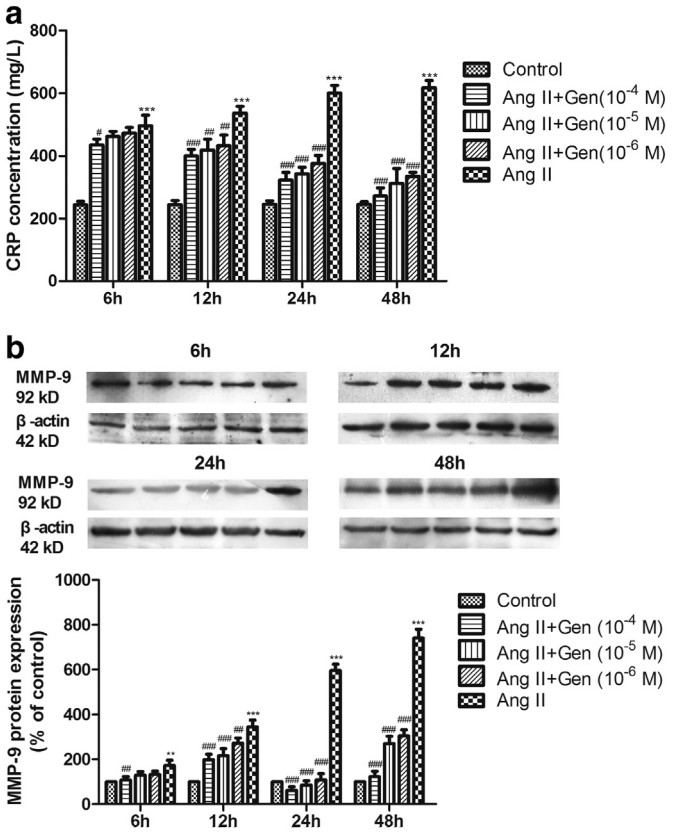

Fig. 2. Effects of genistein on protein expressions of Ang II-stimulated CRP and MMP-9 in VSMCs. VSMCs were pretreated with or without genistein (Gen, 10^{-4}, 10^{-5}, 10^{-6} M) for 6, 12, 24, and 48 h before exposure to Ang II (10^{-7} M) for 24 h. Then, CRP concentration (a) and MMP-9 protein expression (b) were measured by ELISA and western blot respectively. Results from three independent experiments were presented as the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Ang II.

primers used were: CRP, forward 5′-CATCTGTGCCACCTGGGAGTC-3′, reverse, 5′-AAGCCACCGCCATACGAGTC-3′; MMP-9, forward, 5′-CCCT ACTGCTGGTCCTTCTGAG-3′, reverse, 5′-AATTGGCTTCCTCCGTGAT TCG-3′; GADPH, forward, 5′-GCAAGTTCAACGGCACAGTCAAG-3′, reverse, 5′-ACATACTCAGCACCAGCATCACC-3′. After synthesizing the cDNA, the products were amplified for 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. GADPH was used as an endogenous reference. The PCR products were separated by electrophoresis on a 2% agarose gel and data were expressed as a ratio of the target transcript relative to GADPH.

2.7. Statistical analysis

Each experiment was performed at least three times. All the data were expressed as the mean ± standard error of mean (S.E.M). One-way ANOVA followed by Bonferroni's test was applied to analyze the differences between the data sets. Values of $P < 0.05$ were considered statistically significant for ANOVA. The Bonferroni adjusted P values were determined by using the original P value divided by the number of comparisons. The level of significance was set at $P < 0.05$/comparison times. Statistical software SPSS 19.0 was used for the statistical analysis.

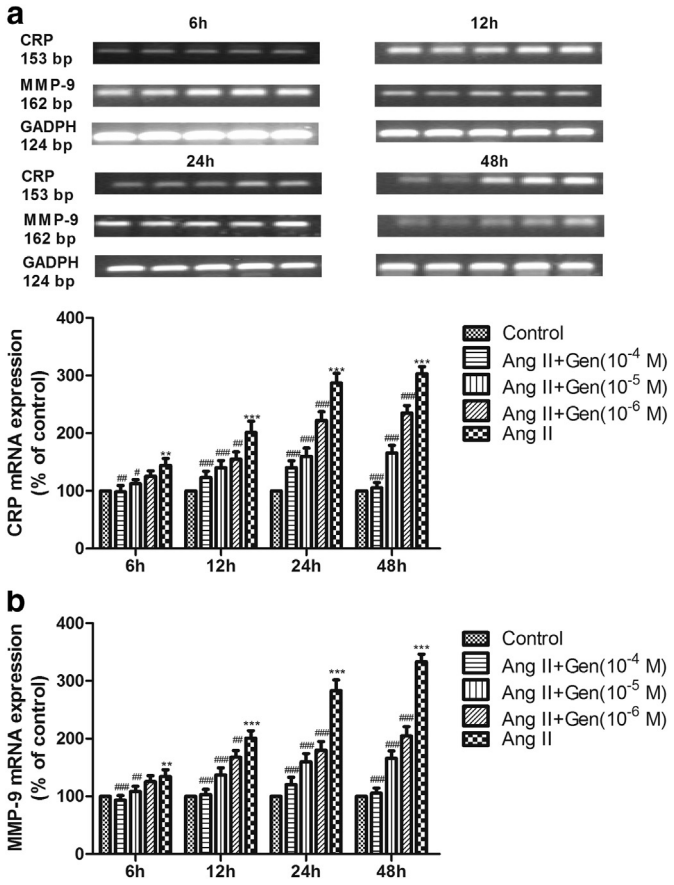

Fig. 3. Effects of genistein on Ang II-stimulated mRNA expressions of CRP and MMP-9 in VSMCs. VSMCs were pretreated with or without genistein (Gen, 10^{-4}, 10^{-5}, 10^{-6} M) for 6, 12, 24, and 48 h before exposure to Ang II (10^{-7} M) for 24 h. Then, mRNA expressions of CRP (a) and MMP-9 (b) were detected by RT-PCR. Results from three independent experiments were presented as the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Ang II.

3. Results

3.1. Effects of Gen on CRP and MMP-9 protein expressions in the non-stimulated VSMCs

Fig. 1 illustrated the effect of Gen on CRP generation and MMP-9 protein expression in the non-stimulated VSMCs. Results showed that there were no significant differences in CRP and MMP-9 generations in Gen-treated groups compared with control group after treatment of the cells with 10^{-6}–10^{-4} M Gen for 6–48 h.

3.2. Effects of Gen on Ang II-stimulated mRNA and protein expressions of CRP and MMP-9 in VSMCs

As shown in Figs. 2 and 3, Ang II increased mRNA and protein expressions of CRP and MMP-9 in VSMCs. However, Gen inhibited Ang II-stimulated mRNA and protein expressions of CRP and MMP-9 in VSMCs in concentration- and time-dependent manners.

3.3. Effects of Gen on Ang II-activated p38/ERK1/2 phosphorylations and NF-κB expression in VSMCs

Results in Fig. 4a, b and c indicated that Ang II significantly increased p-ERK1/2, p-p38 and NF-κB protein expressions in VSMCs, while Gen remarkably attenuated Ang II-induced protein expressions of p-ERK1/2, p-p38 and NF-κB in VSMCs in a concentration-dependent

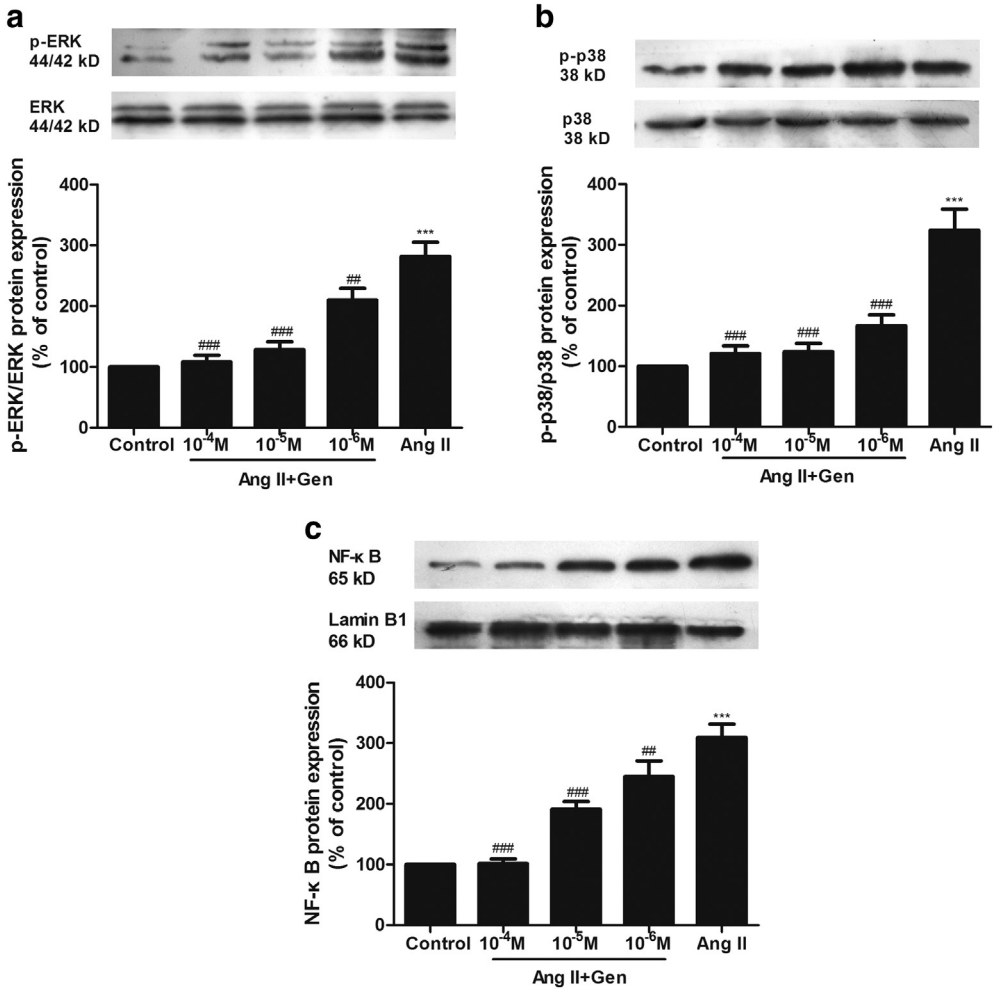

Fig. 4. Effects of genistein on Ang II-activated MAPK phosphorylation and NF-κB expression in VSMCs. VSMCs were pretreated with or without genistein (Gen, 10^{-4}, 10^{-5}, 10^{-6} M) for 24 h before exposure to Ang II (10^{-7} M). Then, protein expressions of p-ERK1/2 (a), p-p38 (b) and NF-κB (c) were analyzed by western blot. Results from three independent experiments were presented as the mean ± S.E.M. $^{***}P < 0.001$ vs the control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Ang II.

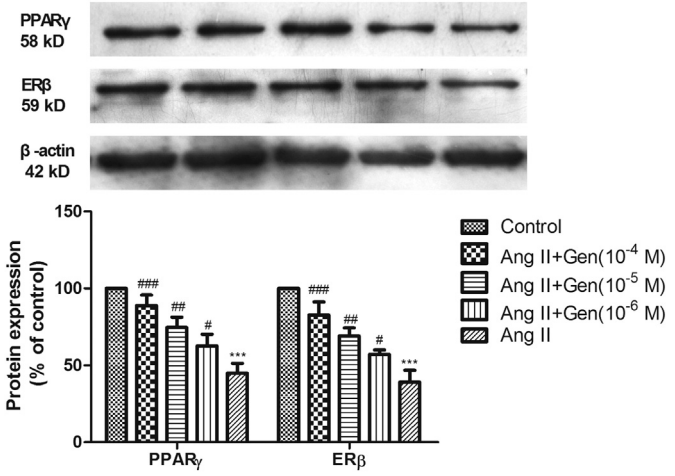

Fig. 5. Effects of genistein on ERβ and PPARγ expressions in Ang II-stimulated VSMCs. VSMCs were pretreated with or without genistein (Gen, 10^{-4}, 10^{-5}, 10^{-6} M) for 24 h before exposure to Ang II (10^{-7} M). Then, ERβ and PPARγ protein expressions were analyzed by western blot. Results from three independent experiments were presented as the mean ± S.E.M. $^{***}P < 0.001$ vs the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs Ang II.

way.

3.4. Effects of Gen on Ang II-downregulated ERβ and PPARγ expressions in VSMCs

Fig. 5 exhibited that Ang II decreased ERβ and PPARγ protein expressions in VSMCs. Compared with Ang II alone, pretreatment of the cells with Gen antagonized ERβ and PPARγ downregulations in a concentration-dependent manner in Ang II-stimulated VSMCs.

3.5. ERβ lies upstream of MAPK in the inhibitory effect of Gen on Ang II-stimulated CRP and MMP-9 expressions in VSMCs

As shown in Fig. 6, Ang II obviously increased p-ERK and p-p38 expressions, whereas Gen significantly diminished Ang II-stimulated ERK1/2 and p38 phosphorylations. Additionally, the ER blocker ICI182780 antagonized the inhibitory effect of Gen on Ang II-stimulated p-ERK1/2 and p-p38 expressions in VSMCs.

3.6. Gen inhibits Ang II-induced CRP generation and MMP-9 expression via the ERβ-PPARγ-NF-κB pathway

Results in Fig. 7a,b,c,d showed that Ang II increased CRP generation and MMP-9 protein expression, while Gen reduced Ang II-stimulated CRP and MMP-9 generations in VSMCs. Furthermore, ERβ blocker ICI182780 and PPARγ blocker GW9662 abolished the inhibitory effects

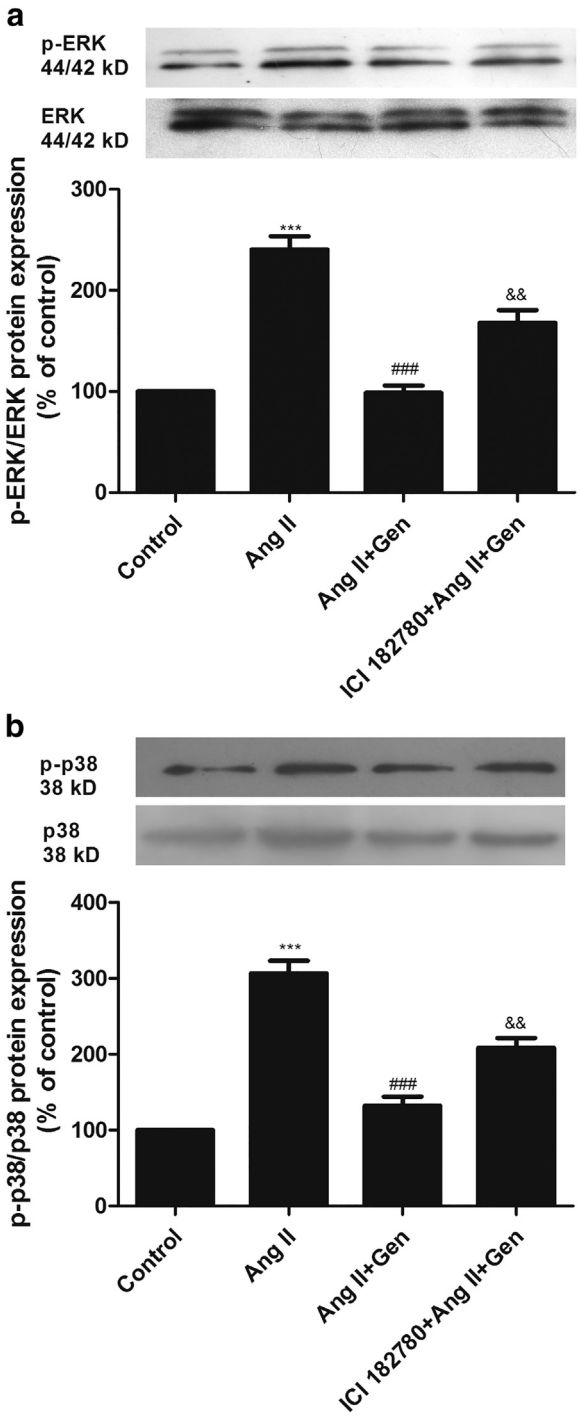

Fig. 6. ERβ lies upstream of MAPK in the inhibitory effect of genistein on Ang II-stimulated CRP and MMP-9 expressions in VSMCs. VSMCs were pretreated with ICI 182780 (1 μM) for 60 min before treatment of genistein (Gen, 10^{-5} M) for 24 h and subsequently stimulated with Ang II (10^{-7} M) for 24 h. The protein expressions of p-ERK1/2 (a) and p-p38 (b) were evaluated by western blot. Results from three independent experiments were presented as the mean ± S.E.M. $^{***}P < 0.001$ vs the control; $^{\#\#\#}P < 0.001$ vs Ang II; $^{\&\&}P < 0.01$ vs Ang II + Gen.

of Gen on Ang II-stimulated CRP and MMP-9 generations in VSMCs.

Fig. 7e indicated that Ang II down-regulated PPARγ expression and Gen up-regulated PPARγ expression in Ang II-stimulated VSMCs. However, pretreatment of the cells with ERβ blocker ICI182780 completely abolished the up-regulated effect of Gen on PPARγ expression in Ang II-stimulated VSMCs.

As seen from Fig. 7f, Ang II increased NF-κB expression and Gen significantly down-regulated Ang II-induced NF-κB expression in VSMCs. Preincubation of the cells with PPARγ blocker GW9662 prior to Gen treatment attenuated the inhibitory effect of Gen on NF-κB expression in Ang II-stimulated VSMCs.

4. Discussion

Atherosclerosis is a chronic inflammatory response of the vascular wall. Inflammation is involved in all phases of atherosclerosis, from initiation, to progression, and finally to plaque rupture. So, anti-inflammatory therapy is a new measure for the prevention and treatment of atherosclerostic diseases. As the most representative inflammatory marker, CRP directly participates in the initiation and development of atherosclerosis by amplifying more inflammation [15]. In addition to CRP, MMP-9 is related to plaque unstability via the degradation of ECM and the regulation of the migration of inflammatory cells.

Gen is the most abundant content of soybean isoflavones and is known to its preventive and therapeutic effects on cardiovascular diseases. The present study showed that Gen inhibited Ang II-stimulated CRP and MMP-9 expressions in transcription and protein levels in VSMCs, suggesting that Gen plays a beneficial role in cardiovascular system through anti-inflammatory effect.

It is well known that MAPK signal pathway is involved in inflammatory response in the cardiovascular diseases. Previous studies reveal that Ang II induces CRP and MMP-9 expressions in VSMCs via the phosphorylations of ERK1/2 and p38 [13,16,17]. The present results demonstrated that Gen significantly diminished Ang II-activated ERK1/2 and p38 phosphorylations. Combinating with the known reports, it is suggested that Gen reduces Ang II-induced CRP and MMP-9 generations in VSMCs by interfering with ERK1/2 and p38 signal pathway.

Gen also exerts a weak estrogenic activity and produces a protection on the cardiovascular system by binding to ERs. ERβ plays a predominant role in the heart and vascular system compared with ERα and mediates a protective effect on the cardiovascular system by inhibiting VSMC migration [18]. Gen has been illustrated to exhibit a considerably stronger affinity for ERβ than for ERα [19]. Moreover, Gen produces an anti-inflammatory effect via the activation of ERβ in the central nervous system [20]. The present experiment displayed that Ang II decreased ERβ expression, which was accordance with Chen's study [21], while Gen antagonized Ang II-downregulated ERβ expression. The further study indicated that ERβ blocker ICI 182780 abolished the inhibitory effect of Gen on Ang II-induced CRP and MMP-9 generations, implying that Gen also exerts an anti-inflammatory effect in Ang II-stimulated VSMCs by up-regulation of ERβ.

In addition to ER in the nucleus, there are partial ERs localized to the plasma membrane [22], which are the same isoforms in purified plasma membranes and in the nucleus. Klinge et al. find that ERs localized at the plasma membrane initiate rapid signal transduction through MAPK [23]. The present results illustrated that the inhibitory effects of Gen on Ang II-stimulated ERK1/2 and p38 phosphorylations were abolished by ER blocker ICI 182780, suggesting that ER may lie upstream of ERK1/2 and p38 in the repressive effects of Gen on CRP and MMP-9 expressions in Ang II-stimulated VSMCs.

PPARγ also plays multiple beneficial effects in treatment of atherosclerosis via the anti-inflammatory effect [24]. It has been proved that Ang II suppresses PPARγ activity [25] and activation of PPARγ reduces the expressions of CRP and MMPs in VSMCs [26,27]. The present study showed that Gen reversed Ang II-downregulated PPARγ expression and PPARγ blocker GW9662 attenuated the inhibitory effect of Gen on CRP and MMP-9 expressions in Ang II-stimulated VSMCs, confirming that Gen inhibits Ang II-stimulated CRP and MMP-9 expressions via activation of PPARγ as well.

Singh reports that PPARγ agonism serves as one of the mechanisms in the estrogen-mediated effects [28]. Our study showed that ER

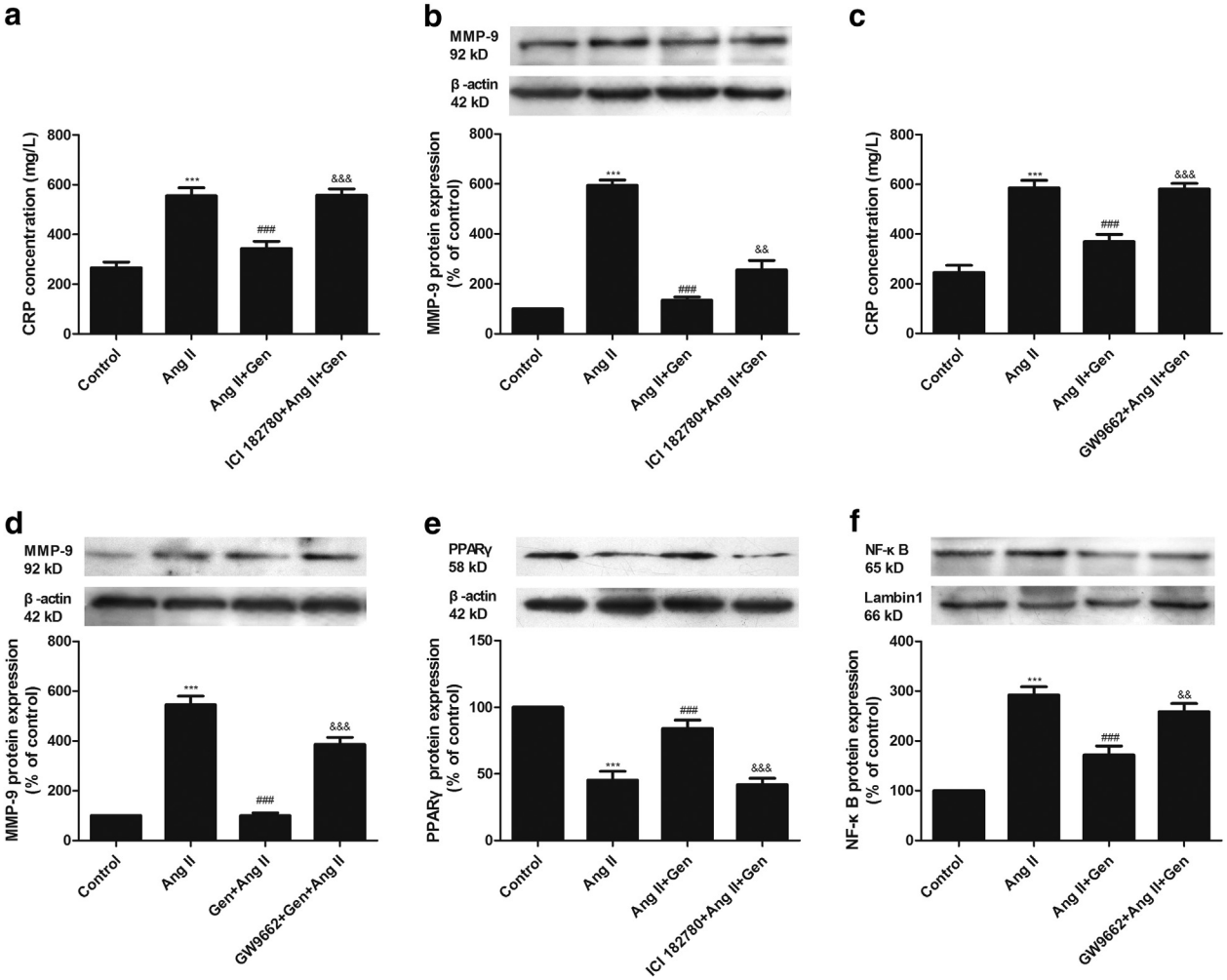

Fig. 7. Genistein inhibits Ang II-induced CRP and MMP-9 expressions via the ERβ-PPARγ-NF-κB pathway. VSMCs were pretreated with ICI182780 (1 μM) or GW9662 (10 μM) for 60 min prior to treatment with genistein (Gen, 10^{-5} M) for 24 h, and then stimulated by Ang II (10^{-7} M) for 24 h. CRP concentration was measured by ELISA (a, c) and MMP-9 protein expression was measured by western blot (b, d). (e) VSMCs were pretreated with ICI182780 (1 μM) for 60 min prior to treatment with Gen (10^{-5} M) for 24 h, and then stimulated by Ang II (10^{-7} M) for 24 h. PPARγ protein expression was analyzed by western blot. (f) VSMCs were pretreated with GW9662 (10 μM) for 60 min prior to treatment with Gen (10^{-5} M) for 24 h, and then stimulated by Ang II (10^{-7} M) for 24 h. NF-κB expression was measured by western blot. Results from three independent experiments were presented as the mean ± S.E.M. $^{***}P < 0.001$ vs the control; $^{\#\#\#}P < 0.001$ vs Ang II; $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ vs Ang II + Gen.

blocker ICI182780 weakened Gen-activated PPARγ, indicating that Gen may activate PPARγ through ERβ. Interestingly, decreasing PPARγ abundance induced by Ang II in cultured VSMCs was via p38 and ERK phosphorylations [25,29]. Since the above-mentioned results conclude that ERβ lies upstream of p38 and ERK, it is inferred that Gen suppresses CRP and MMP-9 expressions via the ERβ-p38/ERK1/2-PPARγ pathway.

NF-κB as a family of transcription factors is a mediator to regulate inflammatory response. The inflammatory response in atherosclerosis is usually dependent on the activity of NF-κB [30]. Multiple reports indicate Ang II induces CRP and MMP-9 expressions in VSMCs through activating NF-κB [13,16]. The present result showed that Gen inhibited Ang II-stimulated NF-κB expression, which suggests that Gen reduces Ang II-stimulated CRP and MMP-9 levels by interfering with NF-κB in VSMCs. It is known that PPARγ agonists exert an anti-inflammation effect via reducing expression of NF-κB [31]. Our result also showed that PPARγ blocker GW9662 antagonized the repressive effect of Gen on Ang II-stimulated NF-κB. These data demonstrate that Gen inhibits Ang II-stimulated NF-κB expression through activating PPARγ.

Based on prior research conclusions and the present results, we demonstrate that Gen inhibits Ang II-stimulated CRP and MMP-9 expressions in VSMCs via the ERβ-p38/ERK1/2-PPARγ-NF-κB pathway.

5. Conclusions

The present study provides new experimental evidence supporting that Gen is beneficial to cardiovascular system via its anti-inflammatory and plaque stabilized effects. The effects are accomplished by the ER-p38/ERK1/2-PPARγ-NF-κB-CRP/MMP-9 signaling pathway.

Acknowledgements

The study was supported by grants from the Social Development Grant of Shaanxi Province, China (No. 2012K15-02-04).

Conflict of interest statement

The authors declare that there are no conflicts of interests.

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