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PTX3, a Key Component of Innate Immunity, Is Induced by SAA via FPRL1-Mediated Signaling in HAECs

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ABSTRACT

Serum amyloid A (SAA) is regarded as an important acute phase protein in coronary artery diseases. However, its involvement in the immune response of atherosclerosis is poorly understood. The present study was designed to investigate the influence of SAA on the secretion of long pentraxin 3 (PTX3), a key component of innate immunity, in human aortic endothelial cells (HAECs). Our study revealed that recombinant SAA up-regulated PTX3 production in a remarkable dose- and time-dependent manner and the activation of formyl peptide receptor-like 1 (FPRL1) was crucial for SAA-induced expression of PTX3 in HAECs. Meanwhile, SAA-induced PTX3 production could be significantly down-regulated by using the specific siRNA sequences for Jun N-terminal kinases (JNK). Furthermore, the activation of activator protein-1 (AP-1) was necessary for the up-regulation of PTX3 expression. We also found that the activation of nuclear factor-kappa B (NF-κB) played an important role in this process. Our findings demonstrate that SAA up-regulates PTX3 production via FPRL1 significantly, and thus, contributes to the inflammatory pathogenesis of atherosclerosis. J. Cell. Biochem. 112: 2097–2105, 2011.

KEY WORDS: SAA; PTX3; FPRL1; JNK; ATHEROSCLEROSIS

Inflammation plays a key role in the pathogenesis of atherosclerosis, so does immunity [Ross, 1999; Libby, 2002; Shoenfeld et al., 2005]. When human tissue is challenged by inflammatory stimuli, several types of proteins that evoke the immune defense are released into the circulation. Serum amyloid A (SAA) is such kind of major acute phase protein that is released into the circulation and the serum concentration of it could be increased by up to 1,000-fold over the basal level in response to major cardiac events [Jousilahti et al., 2001; Katayama et al., 2003]. So far, it has been proved by many studies that SAA can stimulate the secretion of series of inflammatory cytokines, such as INF-γ, IL-6, IL-8, and so on [Furlaneto and Campa, 2000; Koga et al., 2008]. Furthermore, the expression of SAA has been reported to be involved in atherosclerotic lesions such as the process of lipoprotein retention [O'Brien et al., 2005], and the plasmic level of SAA is closely related to the lesion size of atherosclerosis [Lewis et al., 2004]. All of these findings suggest that SAA may be a crucial component in the chronic progression of atherosclerosis. Nevertheless, how SAA works directly on the pathology of atherosclerosis has not been fully illustrated.

There are three known SAA receptors, including formyl peptide receptor like-1 (FPRL1), CD36 and LIMPII analogous-1 (CLA-1), and toll-like receptor 2 (TLR2), among which, the G protein-coupled FPRL1 is responsible for the chemotactic activity of SAA [Su et al., 1999] and for some of the cytokine induction effects of SAA [He et al., 2003; Lee et al., 2006], while the scavenger receptor CLA-1 mediates the cholesterol efflux function of SAA. Also, recent studies indicate that SAA is a potential ligand of TLR2 in inflammatory diseases [Cheng et al., 2008].

Pentraxins, an acute immunological response family of proteins that consists of three members including C reactive protein (CRP), serum amyloid P (SAP), and pentraxin 3 (PTX3), just like SAA, are also classic acute phase reactants that can reflect the levels of inflammation exactly [Breviario et al., 1992; Garlanda et al., 2005]. PTX3, as a cytokine-inducible factor, is mainly produced in vascular endothelial cells, fibroblasts, and some other extrahepatic tissues

Zhe Dong and Fengling An contributed equally to this article.

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[Breviario et al., 1992; Alles et al., 1994; Introna et al., 1996; Goodman et al., 2000; Polentarutti et al., 2000; Abderrahim-Ferkoune et al., 2003; Doni et al., 2003; Klouche et al., 2004], which makes it distinguished from the other two members that are synthesized majorly in the liver [Toniatti et al., 1990; Pepys and Hirschfield, 2003]. Nevertheless, PTX3 can also activate complement and bind to cellular debris, just similar to the classic pentraxins. Immobilized PTX3 activates the classical complement pathway through specific recognition and interaction with the complement component C1q in a calcium-independent manner [Nauta et al., 2003]. In addition, by interacting with P-selectin, PTX3 modulates leukocyte recruitment in inflammation and PTX3 has shown its role as a novel scavenger molecule through binding to apoptotic cells and regulating their clearance [Rovere et al., 2000; Deban et al., 2010]. Recently, PTX3 protein has been found in the advanced atherosclerotic plaques of human beings, which means that it may play an important role in the development of atherosclerosis [Rolph et al., 2002].

As mentioned above, the levels of both SAA and PTX3 are increased in the process of atherosclerosis. Whether there is a close relevance between SAA and PTX3 is worth pondering. Thus, in this study, we aimed to investigate the capacity of human aortic endothelial cells (HAECs) to express PTX3 after pro-incubation with SAA. Then we further discussed the involvement of FPRL1 in SAA-induced PTX3 production, as well as the signaling pathways involved in this process.

MATERIALS AND METHODS

MATERIALS

Recombinant human SAA (Endotoxin level is less than 0.1 ng/μg) was purchased from Peprotec (Rocky Hill, NJ). Recombinant human TNF-α was purchased from R&D (Shanghai, China). Pertussis toxin was purchased from Sigma (Shanghai, China). H2N-WRWWWW-CONH2 (WRW^4), a FPRL1 Antagonist, was purchased from Merck (Shanghai, China). Human FPRL1 siRNA (Santa Cruz, CA, sc-40123) is an effective tool designed to knock down gene expression conveniently. Rabbit anti-human antibodies for FPRL1 and phospho-NF-κBp65 were purchased from Abcam (Cambridge, MA) and rabbit anti-human antibodies for β-actin were purchased from Santa Cruz Biotechnology. The specific siRNA sequences of Jun N-terminal kinases (JNK) were obtained from Genepharma (Shanghai, China). Rabbit anti-human antibodies for JNK1 and mouse anti-human antibodies for JNK2 were purchased from Abcam. Tanshinone IIA which was extracted from Chinese medicinal herb Danshen and could inhibit activator protein-1 (AP-1) activity was purchased from Sigma. BAY 11-7082 and SC-514, two effective NF-κB inhibitors, were purchased from Beyotime Biotech (Jiangsu, China) and Merck(Shanghai, China).

CELL CULTURE

Human aortic endothelial cell line was obtained from ATCC and was cultured in endothelial culture medium (ECM, ScienCell) supplemented with 5% fetal bovine serum. These cells were used for experiments between 3rd and 5th passage [Norata et al., 2003].

SMALL INTERFERENCE RNA (siRNA) AND ITS TRANSFECTION

The specific siRNA sequences of JNK were used as follows: JNK1 5′-GAAGCU CAGCCGGCCAUUU-3′; JNK2 sense, 5′-GUGAACUU-GUCCUCUUAAATT-3′, anti-sense, 5′-UUUAAGAGGACAAGUU-CACTT-3′. FPRL1 siRNA sequences were obtained from Santa Cruz Biotechnology (sc-40123). Transfection of HAECs involved the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The siRNA/lipfectamine 2000 particles were prepared in the presence of effective siRNA (100 nM) and were added onto the HAECs. After 48 h, HAECs were challenged by SAA (10 μg/ml) for another 24 h, and then both cells and supernatants were collected for detection.

QUANTITATIVE REAL-TIME PCR

HAECs were stimulated for different checkpoints with 10 μg/ml of SAA. After being isolated from the HAECs respectively using TRIzol reagent (Invitrogen), the concentrations of total RNA were measured using an UV spectrophotometer (Bio-Rad, Beijing, China). Then 1 μg total RNA was reverse-transcribed with the use of the MMLV Reverse Transcriptase System (Fermentas, Germany) and oligo (dT). The real-time PCR was performed with the use of LightCycler (Roche Applied Science, Mannheim, Germany) following the instructions. SYBR Green I kit (TaKaRa Biotechnology, Kyoto, Japan) was used and the primer sequences for real-time PCR analyses were as follows; PTX3, forward: 5′-TAT TCC CAA TGC GTT CCA AGA A-3′, reverse: 5′-GCC ATA GGA AAA CAG GAT GGT T-3′; FPRL1, forward: 5′-CAC GGC CAC ATT ACC ATT CCT-3′, reverse: 5′-AGC GGT CCA GTG CAA TGA AA-3′; JNK1, forward: 5′-ATG CTA AGC GAG CCT ACC G-3′, reverse: 5′- TCT CAA AGC TAT AGC CAG CG-3′; JNK2, forward: 5′- ACC TCC TCT ACC AGA TGC T-3′, reverse: 5′-TGA ACT CTG CGG ATG GTG-3′; β-actin, forward: 5′-CAT GTA CGT TGC TAT CCA GGC-3′, reverse: 5′-CTC CTT AAT GTC ACG CAC GAT-3′. The specificity of products generated for each set of primers was tested, respectively, by using a melting curve and gel electrophoresis. The relative expression levels of each targeted gene were normalized with the use of the ddC_T comparative method [Yuan et al., 2006]. Each sample was run in triplicate for real-time PCR.

ELISA

HAECs were placed in ECM containing 5% FBS in 96-well plates and kept in a 5% CO_2 incubator at 37°C. After stimulation, cell-free supernatants were collected, centrifuged, and assayed for PTX3 by enzyme-linked immunosorbent assay (ELISA, R&D) according to the manufacturer's instructions [Norata et al., 2008].

WESTERN BLOT

After being stimulated by 10 μg/ml of SAA for 24 h, HAECs were harvested for protein extraction. Extracted proteins were separated in 10% SDS-polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes (Pierce), which was then blocked by incubating with 5% nonfat dry milk in TBS-T [20 mmol/L Tris-HCl (pH 8.0), 8 g/L NaCl, and 0.1% Tween 20] for 2 h at room temperature. After that, membranes were incubated with specific

antibodies at 4°C overnight and washed with TBS-T. Antigen–antibody complexes were visualized after incubating the membrane with 1:10,000 diluted antibodies coupled to horseradish peroxidase with the SuperSignal West Pico enhanced chemiluminescence kit (Pierce) according to the manufacturer's instructions. Immunoreactive bands were quantified by the use of a densitometer analysis system Flurochem 9900-50 (Alpha Innotech, Santa Clara, CA). The expression level of sample was indicated as a ratio of sample to β-actin.

IMMUNOFLUORESCENCE

Cells cultured on glass cover slips were washed with PBS and fixed in 2% paraformaldehyde for 30 min at room temperature, followed by two rinses in PBS. Cells were permeabilized with 0.2% Triton X-100 for 30 min and then blocked in 3% BSA for 30 min at room temperature. After that, cells were incubated with rabbit anti-phospho-NF-κBp65 antibody (diluted 1:100) overnight at 4°C. After three washes for 10 min each with PBS, these cover slips were incubated with FITC conjugated goat anti-rabbit secondary antibody for 1 h at 37°C and washed three times with PBS. Then, immunolabeled cells were counterstained with DAPI to detect cell nuclei. After being washed, these cells were covered with 50% glycerol and stainings were visualized with a fluorescence microscope equipped with a digital camera.

DATA ANALYSIS

Results were expressed as means ± SEM. Statistical comparisons used one-way ANOVA and significance was considered at $P < 0.05$.

RESULTS

SAA UP-REGULATES PTX3 EXPRESSION AT BOTH mRNA AND PROTEIN LEVELS

To investigate the effect of SAA on PTX3 secretion in HAECs, cells were stimulated with SAA (10 μg/ml) and the supernatant concentration of PTX3 was detected by ELISA at various time points (0, 3, 6, 9, 12, 15, and 24 h). Recombinant human TNF-α (10 ng/ml) was used as a positive control. The results demonstrated that SAA could induce PTX3 accumulation in a time-dependent manner and reached its maximal activity at 24 h after stimulation. The same conclusion could be drawn with the administration of TNF-α (Fig. 1A). Then, the cells were treated with different concentration gradients of SAA for 24 h (0–20 μg/ml). As a result, we observed that SAA could induce PTX3 secretion also in a concentration-dependent manner, and achieved its maximal activity at 10 μg/ml (Fig. 1B). These results altogether indicate that the induction of PTX3 by SAA is time- and dose-dependent. At the same time, the effect of SAA on PTX3 at mRNA transcription level was examined via real-time PCR. Our data demonstrated a similar expression in compliance with its protein level, as the mRNA expression of PTX3 increased by the time of stimulation after 3–9 h treatment with SAA (Fig. 1C), which suggested that SAA-induced PTX3 protein synthesis required transcriptional activation.

SAA INDUCES PTX3 PRODUCTION VIA FPRL1

As is known that SAA is a potent agonist of FPRL1, we then sought to investigate the involvement of FPRL1 in the SAA-inducible expression of PTX3. Hence, we assessed via real-time PCR and western blot that the expression of FPRL1 was up-regulated at both mRNA and protein levels after being stimulated by SAA (Fig. 2A and B). After that, pertussis toxin, an antagonist of G protein-coupled receptor, was chosen to detect whether there was an involvement of a G protein-coupled receptor in this process. It was demonstrated that the SAA-induced PTX3 production in HAECs could be dramatically blocked by the pretreatment of pertussis toxin (Fig. 2E and F). Meanwhile, WRW^4, which is a novel specific inhibitor for FPRL1, showed a similar blocking effect as pertussis toxin (Fig. 2E and F). In addition, human FPRL1 siRNA (Santa Cruz, sc-40123) was a convenient tool designed for FPRL1 gene silencing and its effect had been examined carefully (Fig. 2C and D). Then, human FPRL1 siRNA was used to confirm whether SAA-induced PTX3 production in HAECs was mediated by FPRL1. The data also indicated that the SAA-induced PTX3 secretion could be inhibited significantly by siRNA-mediated FPRL1 gene silencing (Fig. 2E and F). All these results strongly support that FPRL1 is likely to be one of the receptors through which SAA mediates its effects.

SAA-INDUCED PTX3 PRODUCTION IN HAECs IS MEDIATED THROUGH JNK PATHWAY

JNK, as a member of mitogen-activated protein kinases (MAPKs), is one of the major down-stream pathways for a variety of extracellular stresses. It has been reported that the activation of JNK was required for the PTX3 expression in human lung epithelial cells [Han et al., 2005]. Therefore, it is imperative to investigate whether the activation of JNK is also necessary for SAA-induced PTX3 expression in the HAECs. To this end, the specific siRNA sequences of JNK1 and JNK2 were synthesized, and the effect of them had been examined by both real-time PCR and western blot (Fig. 3A, B, and C). After being transfected with the JNK1 and JNK2 siRNA sequences, we found that the expression of JNK1 and JNK2 was down-regulated and the SAA-induced PTX3 expression could be partially but significantly reduced (Fig. 3D). These results strongly suggest that JNK pathway is crucial for SAA-induced PTX3 expression in HAECs, and activations of both JNK1 and JNK2 are involved in mediating PTX3 production.

SAA STIMULATES PTX3 PRODUCTION IN HAECs VIA AP-1 ACTIVATION

AP-1 is a well-known transcription factor that regulates a variety of genes expression in response to various stimuli. The activation of AP-1 has been reported to be usually related to the regulation of JNK, and intriguingly, the human PTX3 proximal promoter has been located to include AP-1 binding site [Garlanda et al., 2005]. Therefore, we hypothesized that AP-1 was highly indicated in the up-regulation of SAA-induced PTX3 expression, either from its direct activation of PTX3 promoter, or from its indirect activation of JNK pathway. So, an AP-1 inhibitor Tanshinone IIA was employed to confirm the variation of PTX3 expression. After being incubated with Tanshinone IIA at various dose levels for 24 h, HAECs were exposed to SAA (10 μg/ml) for additional 24 h. We found that

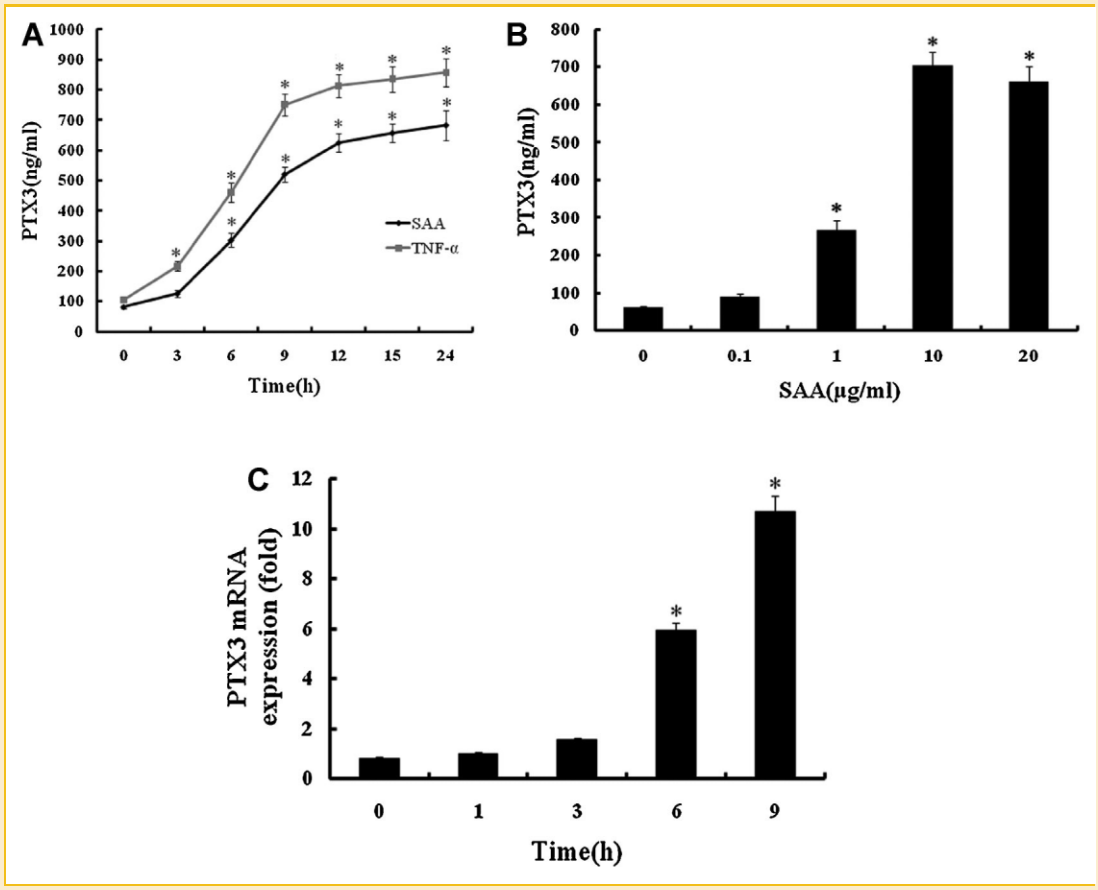

Fig. 1. SAA up-regulates PTX3 secretion in human aortic endothelial cell. A: HAECs were stimulated for different times (0, 3, 6, 9, 12, 15, and 24 h) with 10 μg/ml SAA or 10 ng/ml TNF-α. B: HAECs were stimulated for 24 h with varying concentrations (0, 0.1, 1, 10, and 20 μg/ml) of SAA. In (A–B), PTX3 levels were determined by ELISA. C: HAECs were stimulated for 0, 1, 3, 6, and 9 h with 10 μg/ml of SAA. Then, cells were harvested and total RNA was extracted. Real-time PCR analysis was used for PTX3 mRNA. The data were expressed as means ± SEM and three independent experiments were performed in duplicate. $^{*}P < 0.05$.

Tanshinone IIA could inhibit SAA-induced PTX3 expression dramatically (Fig. 3E), which suggested that the up-regulation of PTX3 need the activation of AP-1.

SAA INDUCES PTX3 PRODUCTION IN HAECs VIA NF-κB ACTIVATION

The expression of the PTX3 gene also requires the activation of NF-κB [Basile et al., 1997]. Thus, we examined the effect of SAA on NF-κB activity in HAECs by using two NF-κB inhibitors, BAY 11-7082, and SC-514. Both BAY 11-7082 and SC-514 are effective in inhibiting the activation of NF-κB (Fig. 4A). Both of them can block SAA-induced PTX3 production significantly in dose-dependent manners (Fig. 4B). These results indicate that NF-κB activation is important for the SAA-induced PTX3 production in HAECs.

DISCUSSION

It is well known that SAA, one of the major acute phase proteins, is significantly increased both systemically in general circulation and locally within impaired tissues as a result of inflammatory response [Fyfe et al., 1997; O'Brien and Chait, 2006]. Thus, SAA is considered as a biomarker that can reflect the inflammatory levels [Jousilahti et al., 2001; Katayama et al., 2003]. Furthermore, it has also been reported as a chemoattractant that could induce migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes [Badolato et al., 1994; Badolato et al., 2000]. More importantly, many studies have shown that the elevated level of SAA could directly stimulate the production of series of cytokines, such as IL-6, IL-10, TF, MCP-1, and so on [Mullan et al., 2006; Zhao et al., 2007; Lee et al., 2008, 2009]. As is discussed above, SAA could be regarded as an important pro-inflammatory mediator in the progression of inflammatory diseases, especially in atherosclerosis. Meanwhile, the elevated secretion of PTX3 in vascular endothelia has been described as an early event in the primary activation of local innate immunity and systemic inflammatory response [Mantovani et al., 2006]. Thus, we hypothesize that the up-regulation of PTX3 may be a result of SAA stimuli, and this needs to be identified. Therefore, it is essential to investigate the influence of SAA on the production of PTX3, which has been confirmed later in our study to be induced by SAA

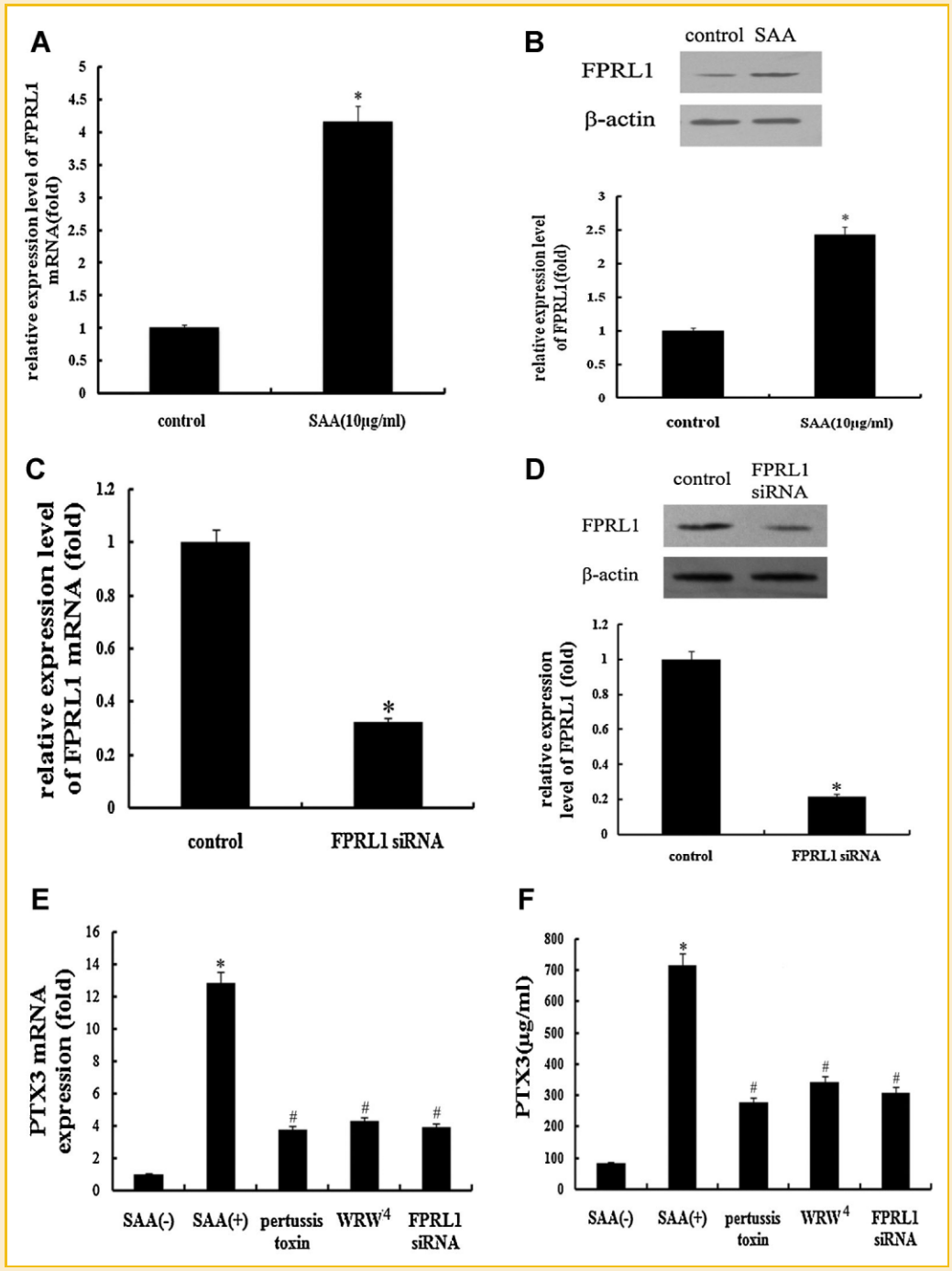

Fig. 2. SAA induces PTX3 production in HAECs via FPRL1. A: After being stimulated with 10 μg/ml SAA for 24 h, real-time PCR showed that SAA up-regulated the expression of FPRL1 mRNA. $^{*}P < 0.05$. B: In the same condition mentioned above, western blot showed that SAA up-regulated the expression of FPRL1 in the protein level. $^{*}P < 0.05$. C–D: After being preincubated with the siRNA sequences of FPRL1 for 48 h, both HAECs and supernatants were harvested for real-time PCR and western blot to check the effect of the siRNA sequences. $^{*}P < 0.05$. E–F: HAECs were preincubated with pertussis toxin (500 ng/ml) or WRW^4 (30 μM) at 37°C for 1 h or human FPRL1 siRNA (100 nM) for 48 h and then stimulated with 10 μg/ml SAA for another 24 h. SAA-induced PTX3 production levels were determined by real-time PCR and ELISA. $^{*}P < 0.05$ vs. SAA (−) group. $\#P < 0.05$ vs. SAA (+) group. The data were expressed as the means ± SEM and were based on three separate experiments.

both in a time- and dose-dependent manner in HAECs. Meanwhile, the capability of SAA on PTX3 production was found to be associated with the accumulation of PTX3 mRNA, which suggests that SAA induces cytokine production at the transcriptional level.

Although PTX3 plasmic level could be regarded as a predictor of 3-month mortality in patients with myocardial infarction, it has been revealed recently to exhibit a cardiovascular protective function by using PTX3-deficient murine models of atherogenesis

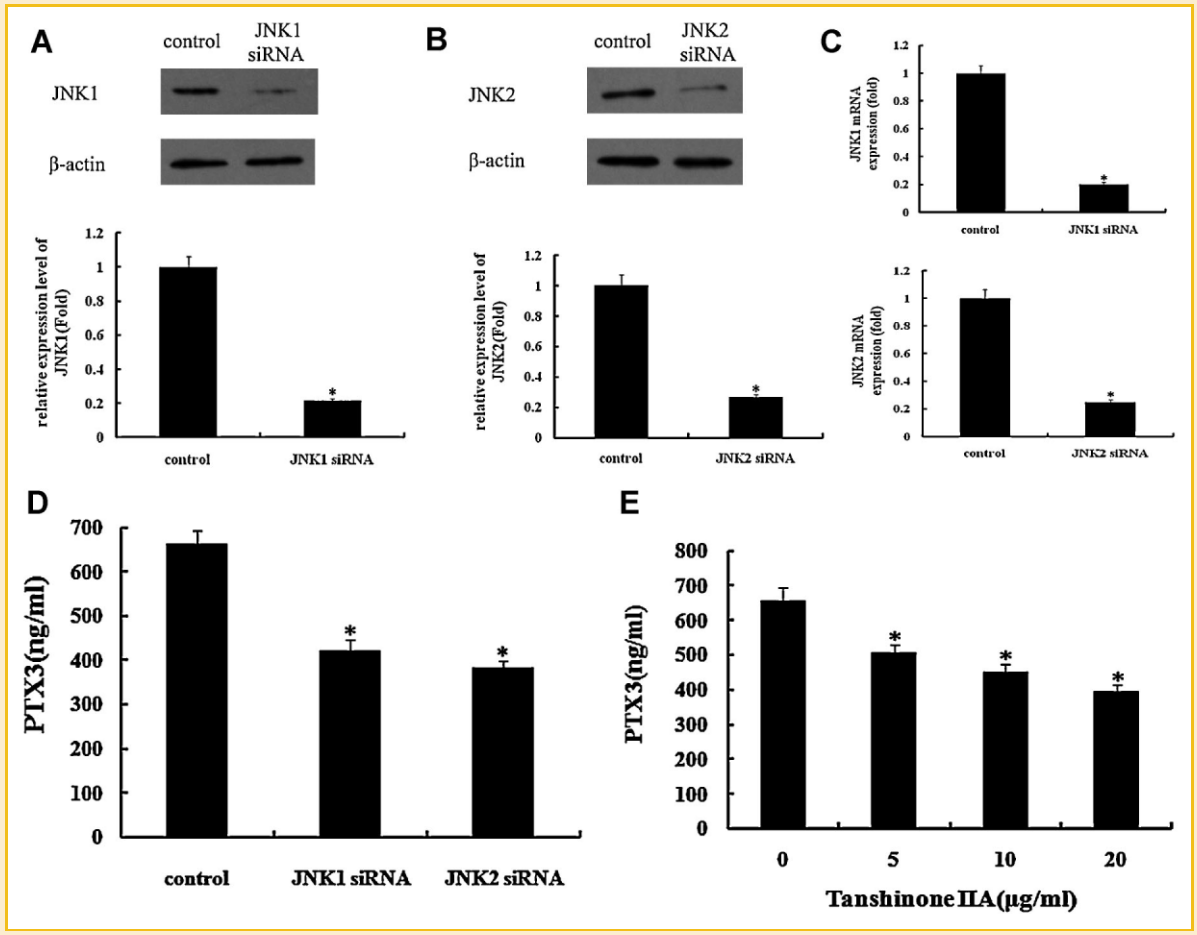

Fig. 3. SAA-induced PTX3 production in HAECs is mediated through JNK pathway. A, B, and C: After being preincubated with the siRNA sequences of JNK1 and JNK2 for 48 h, both HAECs and supernatants were collected for real-time PCR and western blot to check the effect of the siRNA sequences. D: After being preincubated with the siRNA sequences of JNK1 and JNK2 for 48 h, HAECs were stimulated with 10 μg/ml SAA for another 24 h. SAA-induced PTX3 production levels were detected by ELISA. E: SAA-induced PTX3 production can be blocked by Tanshinone IIA. Before being challenged by 10 μg/ml SAA for 24 h, HAECs were incubated with a series of concentrations of Tanshinone IIA (0, 5, 10, 20 μg/ml). Then, cell-free supernatants were collected for ELISA to detect the secreted PTX3 levels. The data were expressed as the means ± SEM and were based on three separate experiments. $^{*}P < 0.05$.

and acute myocardial infarction [Latini et al., 2004; Salio et al., 2008; Norata et al., 2009]. These reports do not contradict the conclusion of our study. Quite the contrary, all of them mean that under the inflammatory stimuli of SAA, the expression of PTX3 can be activated to carry out its protective function through the modulation of the immunoinflammatory balance in HAECs.

Innate immunity is the first line of resistance mechanisms against pathogens, and plays a key role in the activation and orientation of adaptive immunity, as well as the maintenance of tissue integrity and repair. During the procedures of immune response, it is an initial step for our body to recognize the pathogens or damaged tissues, which is predominantly mediated by pattern recognition receptors (PRRs), such as formyl peptide receptors [Garlanda et al., 2005]. Since FPRL1 is known as a potent receptor of SAA, we then sought to focus on its influence on SAA-induced PTX3 expression. It has been proved that FPRL1 is one of the receptors of SAA in this process by using FPRL1 siRNA sequences and WRW^4, an effective inhibitor of FPRL1. The signaling pathway by which SAA regulates such expression has also been discussed. Since related studies indicate that PTX3 expression needs the activation of JNK, whether JNK activation is necessary to SAA-induced PTX3 production has also been explored [Han et al., 2005]. Furthermore, an intensive study has been done to research whether the human PTX3 proximal promoter contains AP-1, NF-κB binding sites [Garlanda et al., 2005]. Our data show that the activations of JNK, AP-1, and NF-κB are crucial for the SAA-induced PTX3 production. These results may provide some novel targets for intervention in this process.

As a whole, our study provides the evidence for the first time that SAA stimulates the secretion of PTX3 via the activation of FPRL1 in HAECs. According to our results, it should be highlighted that SAA may be not only a prognostic indicator but also a pro-inflammatory mediator by inducing the expression of the PTX3, which suggests

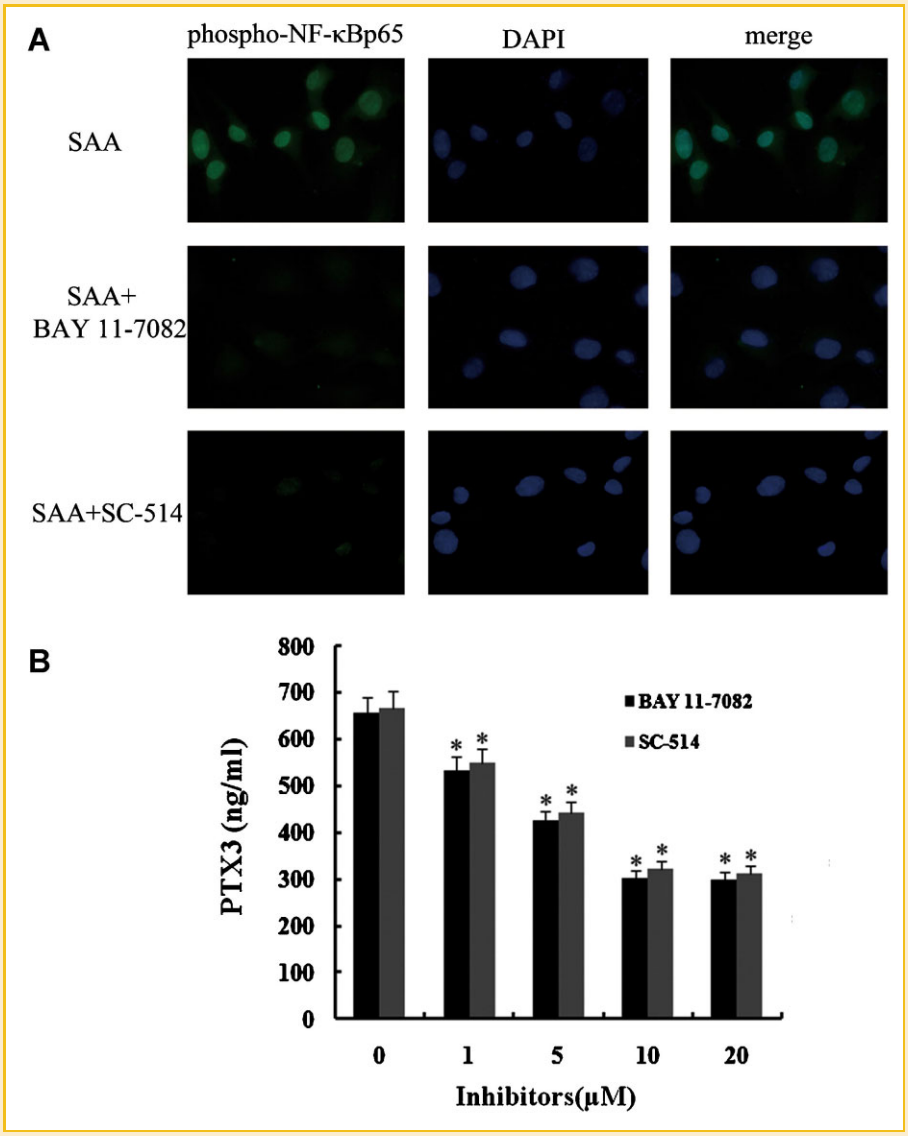

Fig. 4. SAA induced PTX3 production in HAECs via NF-κB activation. A: HAECs were preincubated with BAY 11-7082 (20 μM) and SC-514 (20 μM) at 37°C for 1 h and then stimulated with 10 μg/ml SAA for 24 h. After that, the cells were used for immune-fluorescence staining to observe the inhibited role of BAY 11-7082 and SC-514 on NF-κB activation. B: HAECs were preincubated with a series of concentrations of BAY 11-7082 and SC-514 (0, 1, 5, 10, and 20 μM) at 37°C for 1 h and then stimulated with 10 μg/ml SAA for 24 h. Secreted PTX3 levels were tested by ELISA. The data were expressed as the means ± SEM and $^{*}P < 0.05$.

that SAA could be a potential therapeutic target in the treatment of atherosclerosis.

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