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Activation of spleen tyrosine kinase is required for TNF-α-induced endothelin-1 upregulation in human aortic endothelial cells

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ABSTRACT

Endothelin-1 (ET-1) promotes atherosclerosis. We tested whether spleen tyrosine kinase (Syk) mediates tumor necrosis factor-α (TNF-α)-induced ET-1 upregulation in human aortic endothelial cells (HAECs) and sought to identify the signal pathways involved. TNF-α-induced reactive oxygen species (ROS) activated Syk and phosphatidylinositol 3-kinase (PI3K), which was required for the activation of AP-1 and subsequent ET-1 gene transcription. ROS mediated c-Jun NH_2-terminal kinase (JNK) is also required for AP-1 activation, but Syk and PI3K regulated AP-1 activation independently of JNK. Through regulation of ET-1 production, Syk could be implicated in atherosclerosis.

1. Introduction

Endothelin-1 (ET-1) is implicated in the development of atherosclerosis [1]. Atherosclerosis begins with entrapment of atherogenic lipoproteins to the proteoglycans in the subendothelium of large arteries, which induces inflammatory responses [2]. During this process, pro-inflammatory cytokines are released from interactions among macrophages recruited from the circulation and the cells comprising the vascular wall [3]. In vitro studies have shown that pro-inflammatory cytokines stimulate endothelial cells to secrete ET-1 [4]. ET-1 acts on the vascular smooth muscle cells in a paracrine manner [5], and increases cell proliferation and production of extracellular matrix [6,7], including proteoglycans with longer glycosaminoglycan chains that have higher binding affinity to low density lipoprotein and, therefore, may cause further entrapment of atherogenic lipoproteins [8] leading to progression of the atherosclerotic lesion. In the animal models, ET-1 mRNA expression was elevated in the arteries of atherosclerotic lesion [9], while antagonism of the ET-1 receptors reduced atherosclerotic lesion formation [10,11]. Thus, ET-1 is considered to be one of the therapeutic targets for atherosclerosis [12].

In a previous study of cultured human aortic endothelial cells (HAECs) [13], we demonstrated that tumor necrosis factor-α (TNF-α) stimulates ET-1 production through reactive oxygen species (ROS)-dependent c-Jun NH_2-terminal kinase (JNK) and ROS-independent p38 mitogen-activated protein kinase (MAPK) that regulate both activator protein-1 (AP-1) and nuclear factor κB (NF-κB). Scavenging ROS by antioxidant attenuated TNF-α-induced ET-1 production.

Spleen tyrosine kinase (Syk), a non-receptor protein tyrosine kinase, is a key mediator of Fc and B-cell receptor signaling in inflammatory cells, such as B-cells, mast cells, macrophages and neutrophils [14]. In T and B cells, Syk was activated by oxidative stress as well as antigen receptor engagement [15,16]. Phosphatidylinositide 3-kinase (PI3K) was one of the signal transmitters downstream to Syk that was activated by ROS [17]. Preclinical study demonstrated that Syk inhibitor reduce inflammatory response and ameliorate clinical symptoms of chronic inflammatory diseases including allergic disease [18], rheumatoid arthritis [19] and systemic lupus erythematosis [20].

Other studies have revealed that Syk is also expressed in non-hematopoietic cells such as hepatocytes [21], synoviocytes [22] and vascular endothelial cells [23]. In the synoviocytes [22] and hematopoietic cells [24], Syk was shown to be activated by

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TNF-α. Because atherosclerosis is an inflammatory process in which pro-inflammatory cytokines and ROS are involved, and Syk may have a role in its pathogenesis. In a recent study [25] of low-density lipoprotein receptor-deficient mice, an oral spleen tyrosine kinase inhibitor was shown to attenuate atherogenesis.

In the present study, we evaluated whether Syk is implicated in the TNF-α-induced ET-1 upregulation in human aortic endothelial cells (HAECs). In addition, we investigated how Syk regulates the intracellular signal transduction pathways for TNF-α-induced ET-1 gene expression.

2. Materials and methods

2.1. Materials

TNF-α was obtained from R&D Systems (Abingdon, UK). *N*-acetylcysteine (NAC) and Bay 61-3606 were purchased from Sigma (St. Louis, MO, USA). Syk inhibitor 574711 and SP600125 were from EMD Chemicals Inc (Darmstadt, Germany). LY 294002 and wortmannin were from Alexis biochemicals (San Diego, CA, USA).

Syk-siRNA, control-siRNA, and antibodies to human p65, actin, PI3K, Syk, JNK, ERK, TGF-β1, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to human phospho-JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204) were from Cell Signaling Technology (Danvers, MA, USA). Antibody to human phospho-Syk (pY525/526) was purchased from Epitomics (Burlingame, CA, USA). Anti-phosphotyrosine (4G10) antibody was from Upstate USA, Inc. (Chicago, IL, USA).

2.2. Cell culture

HAECs were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA). The experiments were performed on cells between passages 4 and 6. Before each experiment, the cells were rested for 16 h in RPMI medium (Gibco BRL, Grand Island, NY, USA) containing 2% fetal bovine serum. Prior to the addition of various cell signal inhibitors and TNF-α, the medium was replaced with serum-free RPMI medium.

2.3. Et-1 ELISA

Serum-starved HAECs were untreated or pretreated with Bay 61–3606 for 30 min and then incubated with TNF-α for 24 h. The ET-1 released in cell culture supernatant was quantified by sandwich ELISA (QuantiGlo® human ET-1, R&D Systems, Abingdon, UK) according to the manufacture's instruction. Each experiment was performed in duplicates.

2.4. Real time reverse transcriptase-polymerase chain reaction (RT-PCR) of ET-1 mRNA

Serum-starved HAECs were untreated or pretreated with NAC, Syk inhibitors or PI3K inhibitors for 30 min and then incubated with TNF-α for 12 h. Total RNA was extracted and reverse transcribed to cDNA with a First Strand cDNA synthesis kit (Fermentas Life Sciences, Burlington, ON, Canada) using random hexamer primers. The mRNA levels of preproET-1 and β-actin were analyzed by quantitative real-time PCR using the SYBR Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 7000 sequence detection system (Applied Biosystems). The primers used were as follows; preproET-1, 5′-TGCTCGTCCCTGAT GGATAA-3′ (forward) and 5′-TCACGGTCTGTTGCCTTTGT-3′ (reverse); β-actin, 5′-CCCAAAGTTCACAATGTGGC-3′ (forward) and 5′-AAGTGGGGTGGCTTTTAGGA-3′ (reverse). In each assay, we included a relative standard curve of four serial dilutions of cDNA. Fold changes of preproET-1 and β-actin mRNAs in the experimental samples relative to the control sample were calculated from the cycle threshold numbers by interpolation on a standard curve for each gene. Relative ET-1 gene expression was represented as the fold change in preproET-1 mRNA corrected for that of β-actin mRNA.

2.5. Preparation and transfection of decoy oligodeoxynucleotides (ODNs)

The phosphorothioate double stranded ODNs against the NF-κB and AP-1 binding site and the mismatched ODNs were prepared by Bioneer Co. (Daejeon, Korea), based on the literature [26]. The sequences of ODNs are as follows: NF-κB decoy ODN, 5′-AGTTG AGGGGACTTTCCCAGGC-3′, mismatched NF-κB decoy ODN, 5′-AG TTGAGGCGACTTTCCCAGGC-3′; AP-1 decoy ODN, 5′-AGCTTGTGA GTCAGAAGCT-3′, mismatched AP-1 decoy ODN, 5′-AGCTTGAAT CTCAGAAGCT-3′. The double stranded ODNs were prepared from complementary single-stranded phosphorothiolate-bonded oligonucleotides. Transfection of ODNs was performed to cells grown in 100 mm dish to 80% confluence. DNA was pre-complexed with the opti-MEM reagents (GIBCO BRL, Grand Island, NY, USA) at room temperature for 15 min, and then mixed and incubated with diluted lipofectamine reagent (Life Technologies, Rockville, MD, USA) for 15 min at room temperature. Thereafter, the reagent complex was added to each dish containing fresh medium, and incubated at 37 °C at 5% CO_2. After 6 h incubation, complete medium with serum for normal growth was added. After 2 days transfection period, the cells were subjected to the experiment for real-time RT-PCR.

2.6. Electrophoretic mobility shift assay (EMSA)

Serum-starved HAECs were untreated or pretreated with NAC, Syk inhibitors or PI3K inhibitors for 30 min and then incubated with TNF-α for 30 min. After incubation periods, nuclear extract was prepared. NF-κB and AP-1 consensus oligonucleotides (5′-AG TTGAGGGGACTTTCAGGA-3′ and 5′-CGCTTGATGAGTCAGCCGGAA-3′ respectively) (Promega, Madison, WI, USA) were end-labeled with γ-[32P]-ATP (50 μCi at 3000 Ci/mmol, New England Nuclear, Boston, MA, USA) with T4 polynucleotide kinase. Nuclear protein (10 μg) was allowed to react for 15 min with ^{32}P-labeled NF-κB or AP-1 oligonucleotides (1.75 pmol) in 25 μL of binding buffer. Nucleoprotein–oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel. Thereafter, the gel was dried and DNA–protein complexes were localized by autoradiography for 6–12 h. For the competitive assay, 100 times excess unlabeled NF-κB or AP-1 oligonucleotides were added in the reaction mixture before adding the ^{32}P-labeled NF-κB or AP-1 oligonucleotides.

2.7. Western blot analysis

Treated cells were washed with PBS, and incubated with 100 μl of lysis buffer containing 50 mM KCl, 25 mM HEPES (pH 7.8), 0.5% Igepal CA-630, 1 mM PMSF, 2 μM leupeptin, 1 μM aprotinin and 100 μM DTT on ice for 5 min. For the experiment using nuclear extract, cytosolic fractions were separated using pipette after centrifugation and the remaining nuclear fractions were lysated again with lysis buffer. After repeated freezing and thawing with liquid nitrogen and water bath set to 37 °C, the nuclear lysates were incubated at 4 °C for 20 min with a rocking platform. Finally, nuclear protein was obtained after centrifugation. In other experiments, whole cell lysates were used. Equal amounts of

protein extracts were separated by 12% SDS–polyacrylamide gel electrophoresis, and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The membrane was probed with the antibody directed against human p65, actin, phospho-Syk (Try525/526), Syk, phospho-JNK (Thr183/Tyr185), JNK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-ERK1/2 (Thr202/Tyr204), or ERK. Bands were visualized using horseradish peroxidase conjugated anti-rabbit IgG (Santa Cruz Biotechnology).

2.8. Measurement of ROS

The production of intracellular ROS was evaluated with the probe 5-(and-6)-chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM–H_2DCF–DA; Molecular Probes, Eugene, OR, USA). This reagent enters into cells, and reacts with ROS to generate the fluorescent product 2′-7′-dichlorofluorescein. Serum-starved HAECs were untreated or pretreated with NAC, Syk inhibitors or PI3K inhibitors for 30 min and then incubated with TNF-α for 30 min. Thereafter, the cells were incubated with 5 μmol/L of H_2DCF-DA for 10 min at 37 °C. After removal of the media and wash of the cells, the expression of intracellular ROS was visualized with a LEICA DM-IRE2 inverted microscope (Leica microsystem GmbH, Wetzlar, Germany) equipped with Leica TCS-SP2 confocal system (excitation 488 nm, emission 520 nm). The fluorescent intensities were measured using image-analysis software (Image-Pro® Plus; MediaCybernetics, Bethesda, MD, USA).

2.9. Immunoprecipitation

Equal amounts (100 μg) of whole cell lysates were immunoprecipitated by addition of antibody to PI3K (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immune complexes were recovered by addition of protein A-agarose (Roche Applied Science, Indianapolis, IN, USA) and analyzed by Western blotting with anti-phosphotyrosine (4G10) antibody.

2.10. Transfection of small interfering RNA (siRNA)

Transfection of siRNA directed against Syk (Syk-siRNA) or a non-specific, scrambled, control siRNA (control-siRNA) was also performed using lipofectamine reagent. After transfection of 100 pmol siRNA, the cells were maintained with complete medium for 3 days and then serum-starved for 16 h and subjected to the experiments.

2.11. Statistical analysis

Data are presented as mean ± S.E.(standard error), with n representing the number of different experiments. A Student's t-test was used to compare two values, and an analysis of variance (ANOVA) followed by Dunnett multiple-comparisons test was used to compare mean values among three or more groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TNF-α-induced ET-1 production

HAECs were treated TNF-α (1 ng/ml) for 15 min, 30 min, 1 h, 6 h, 12 h, and 24 h. ET-1 production was measured in the supernatant. TNF-α increased ET-1 production in a time-dependent manner (Fig. 1A). In another experiment, HAECs were treated with different doses of TNF-α (0, 0.1, 1, 2 and 10 ng/ml) for 24 h and ET-1 production was measured. TNF-α increased ET-1 production in a dose-dependent manner (Fig. 1B).

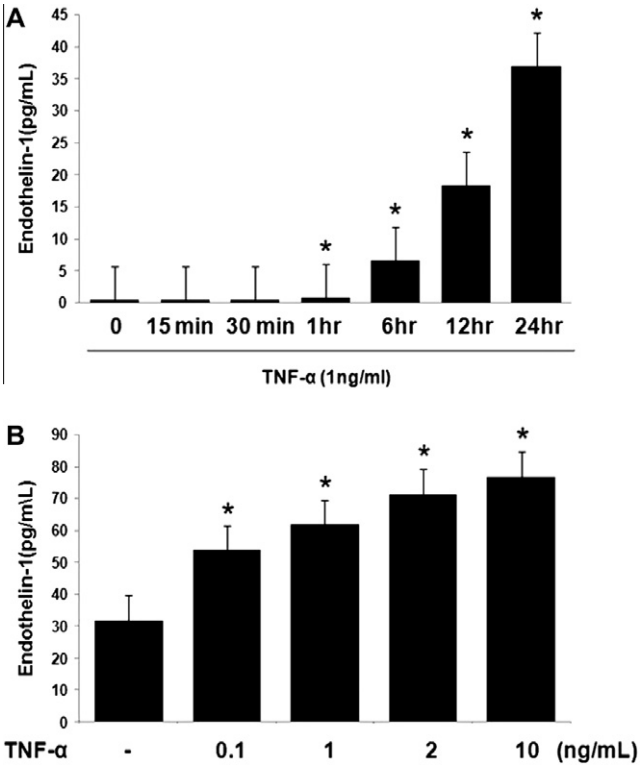

Fig. 1. TNF-α-induced ET-1 production. (A) HAECs were seeded on each well of 24 well plates and cultured for 24 h, and then were serum-starved for 16 h. The cells were treated TNF-α (1 ng/ml) for 15 min, 30 min, 1 h, 6 h, 12 h, and 24 h. ET-1 in the supernatant was measured by ELISA. (B) In another experiment, HAECs were treated with different doses of TNF-α (0, 0.1, 1, 2 and 10 ng/ml) for 24 h and ET-1 in the supernatant was measured. (*$P < 0.05$ compared with control; $n = 4$).

3.2. Syk mediates TNF-α-induced ET-1 production

ET-1 protein level was measured in the supernatant after the HAECs were pretreated with/without Bay 61-3606 (1 or 10 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 24 h. TNF-α significantly increased ET-1 production as compared with control (each $n = 6$, $P < 0.05$). In contrast, TNF-α-induced ET-1 production was effectively attenuated by pretreatment with Bay 61-3606 in a dose-dependent manner (Fig. 2A).

Stimulation of HAECs with TNF-α (1 ng/ml) for 12 h resulted in a significant increase in preproET-1 mRNA. Pre-incubation with Bay 61-3606 (1 μM) or Syk inhibitor 574711 (1 μM) attenuated TNF-α-induced elevation in preproET-1 mRNA (Fig. 1B). NAC (5 mM) also inhibited TNF-α-induced preproET-1 at mRNA levels (Fig. 2B). To see the effect of specific depletion of Syk protein on TNF-α-induced preproET-1 mRNA, we transfected Syk-siRNA or control-siRNA into the cells before treatment with TNF-α. As shown in Fig. 2C, Syk-siRNA effectively inhibited TNF-α-induced preproET-1 mRNA expression.

3.3. PI3K mediates TNF-α-induced ET-1 production

We investigated whether PI3K is also involved in TNF-α-induced ET-1 production. As shown in Fig. 3, both LY 294002 (25 μM) and wortmannin (0.1 μM) significantly inhibited TNF-α-induced preproET-1 mRNA expression.

3.4. Syk and PI3K regulate TNF-α-induced activation of AP-1

To assess whether activation of AP-1 or NF-κB is implicated in TNF-α-induced ET-1 gene transcription, the cells were transfected

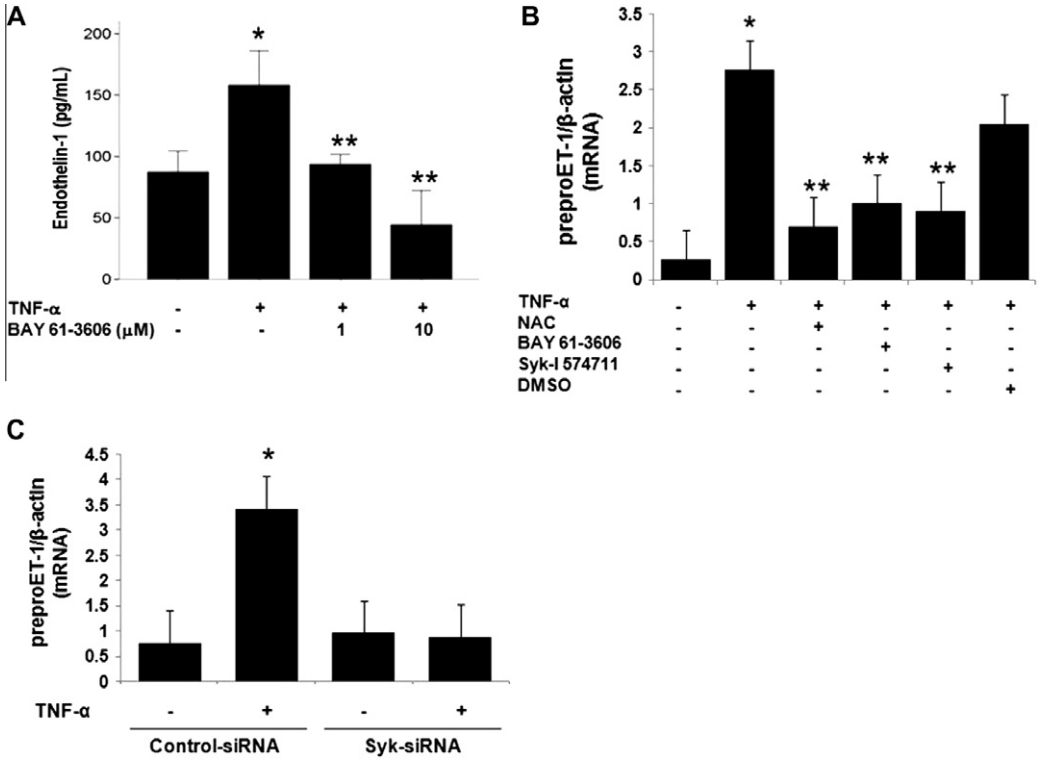

Fig. 2. Syk mediates TNF-α-induced ET-1 production. (A) Effect of Syk inhibitor on TNF-α-induced ET-1 production. HAECs were seeded on each well of 24 well plates and cultured for 24 h, and then were serum-starved for 16 h. The cells were pretreated with/without Bay 61-3606 (1 and 10 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 24 h. The supernatants were collected and ET-1 was measured by ELISA. (*$P < 0.05$ compared with control; **$P < 0.05$ as compared with cells stimulated with TNF-α; $n = 6$). (B) Effects of NAC or Syk inhibitors on TNF-α-induced preproET-1 mRNA expression. Serum-starved HAECs were pretreated with/without NAC (5 mM), Bay 61-3606 (1 μM), Syk inhibitor 574711 (1 μM) or vehicle (DMSO) for 30 min and then incubated with TNF-α (1 ng/ml) for 12 h. PreproET-1 mRNA levels were measured by real-time RT-PCR and expressed as fold induction after normalization to β-actin. (*$P < 0.05$ compared with control; **$P < 0.05$ compared with TNF-α; $n = 8$). (C) Syk-siRNA attenuates TNF-α-induced preproET-1 mRNA expression. Cells were transfected with Syk-siRNA or control siRNA and then treated with TNF-α (1 ng/ml) for 12 h. PreproET-1 mRNA levels were measured by real-time RT-PCR and expressed as fold induction after normalization to β-actin. (*$P < 0.05$ compared with control; $n = 8$).

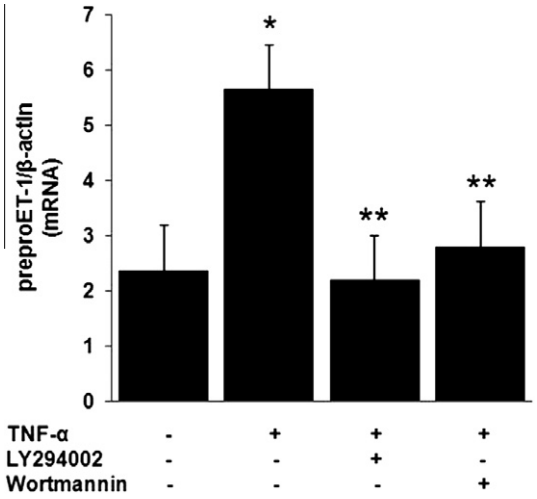

Fig. 3. PI3K mediates TNF-α-induced ET-1 production. Serum-starved HAECs were pretreated with/without LY 294002 (25 μM) or wortmannin (0.1 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 12 h. PreproET-1 mRNA levels were measured by real-time RT-PCR and expressed as fold induction after normalization to β-actin. (*$P < 0.05$ compared with control; **$P < 0.05$ compared with TNF-α; $n = 8$).

with decoy ODNs or mismatched ODNs, and then stimulated with TNF-α. Either AP-1 or NF-κB decoy ODNs significantly inhibited TNF-α-induced preproET-1 mRNA expression as compared with mismatched decoy ODNs (Fig. 4A). In the EMSA, TNF-α increased DNA binding activities of AP-1 and NF-κB, whereas NAC (5 mM) downregulated TNF-α-induced DNA binding activities of both AP-1 and NF-κB (Fig. 4B and C).

To determine whether Syk or PI3K are linked to DNA-binding activity of AP-1 or NF-κB, the cells were pretreated with or without Syk inhibitors and PI3K inhibitors for 30 min and the incubated with TNF-α. Both Syk inhibitors (Bay 61–3603 and Syk inhibitor 574711) and PI3K inhibitors (LY 294002 and wortmannin) downregulated TNF-α-induced DNA binding activity of AP-1 (Fig. 4B), but not NF-κB (Fig. 4C).

We next evaluated the effect of NAC, Syk inhibitors and PI3K inhibitors on the dynamics of p65 protein. The cells were pretreated with/without NAC (5 mM), Bay 61–3606 (1 μM), Syk inhibitor 574711 (1 μM), LY 294002 (25 μM) or wortmannin (0.1 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 30 min, and nuclear extracts from the cells were evaluated. All the inhibitors including NAC did not alter TNF-α-induced nuclear translocation of p65 (Fig. 4D).

3.5. ROS is upstream to Syk and PI3K

TNF-α increased intracellular ROS generation. NAC, an antioxidant, scavenged TNF-α-induced ROS (Fig. 5). In contrast, Syk inhibitors (BAY 61-3606 and Syk inhibitor 574711) as well as PI3K inhibitors (LY 294002 and Wortmannin) had no effect on TNF-α-induced ROS generation.

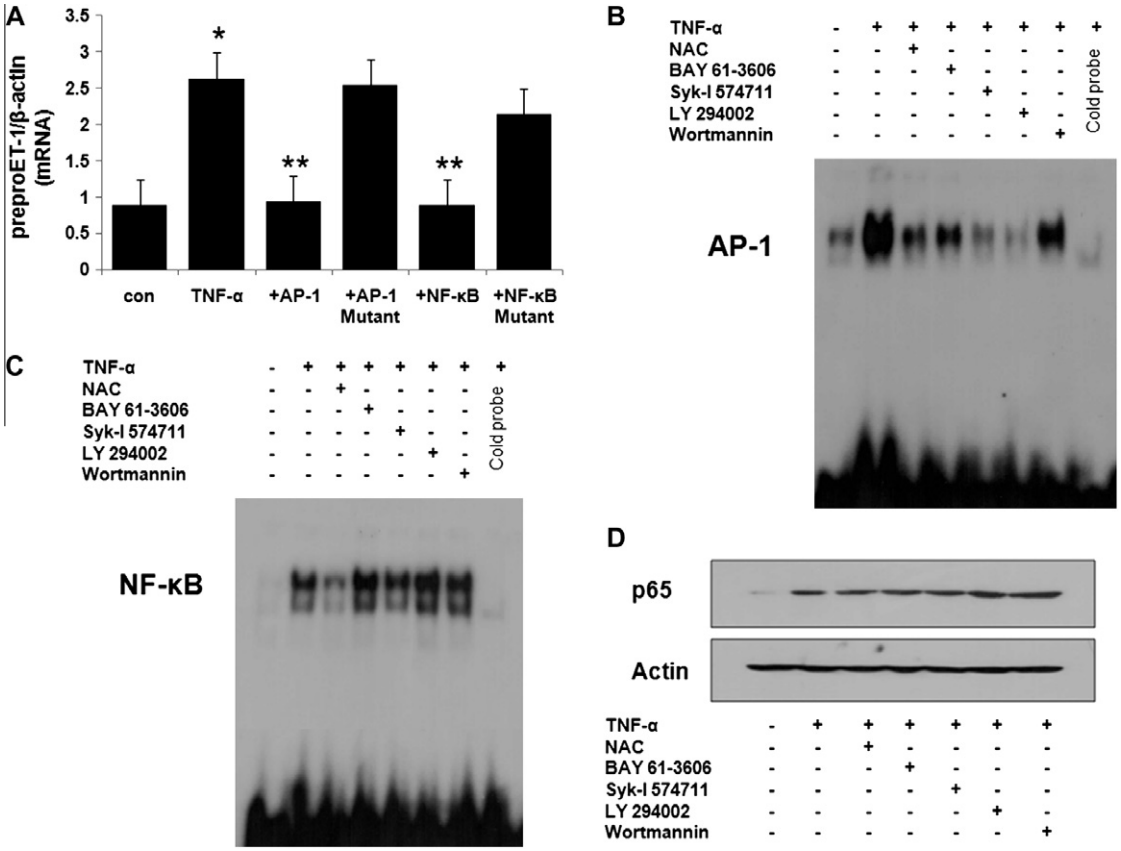

Fig. 4. Syk and PI3K regulate TNF-α-induced activation of AP-1, but not NF-κB. (A) Involvement of AP-1 and NF-κB in TNF-α-induced preproET-1 mRNA expression. Cultured HAECs were transfected with AP-1 or NF-κB decoy oligodeoxynucleotides, or mismatched oligodeoxynucleotides. After serum-starvation, the cells were incubated with TNF-α (1 ng/ml) for 12 h. PreproET-1 mRNA levels were measured by real-time RT-PCR and expressed as fold induction after normalization to β-actin. ($^{*}P < 0.05$ compared with control; $^{**}P < 0.05$ compared with TNF-α; $n = 8$). (B, C) Effects of NAC, Syk inhibitors or PI3K inhibitors on the TNF-α-induced DNA binding activities of AP-1 (B) and NF-κB (C). Serum-starved HAECs were treated with/without NAC (5 mM), Bay 61-3606 (1 μM), Syk inhibitor 574711 (1 μM), LY 294002 (25 μM) or wortmannin (0.1 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 30 min. The nuclear extracts were assayed for the ability to bind ^{32}P-labeled AP-1 or NF-κB oligonucleotides by EMSA. To determine the specificity of the band, 100 times excess unlabeled AP-1 or NF-κB oligonucleotides was added in the reaction mixture before adding the ^{32}P-labeled AP-1 or NF-κB oligonucleotides. The results shown are representative of three independent experiments. (D) Effects of NAC, Syk inhibitors or PI3K inhibitors on the TNF-α-induced nuclear translocation of p65. Serum-starved HAECs were treated with/without NAC (5 mM), Bay 61-3606 (1 μM), Syk inhibitor 574711 (1 μM), LY 294002 (25 μM) or wortmannin (0.1 μM) for 30 min and then incubated with TNF-α for 30 min. The nuclear lysates were analyzed by western blotting using anti-p65 antibody. The result shown is representative of three independent experiments.

3.6. Syk interacts with PI3K

To investigate the relation between Syk and PI3K, HAECs were preincubated with Syk inhibitors or transfected with Syk-siRNA, and then TNF-α-induced tyrosine phosphorylation of PI3K was assessed. After treatment with TNF-α (1 ng/ml) for 15 min, whole cell extracts were obtained and immunoprecipitated with anti-PI3K antibody, and then subjected to Western blot using anti-phosphotyrosine antibody. As shown in Fig. 6A, Bay 61-3606 (1 μM) or Syk inhibitor 573711 (1 μM) attenuated TNF-α-induced tyrosine phosphorylation of PI3K. In addition, Syk-siRNA also inhibited TNF-α-induced tyrosine phosphorylation of PI3K (Fig. 6B). Activation of Syk requires phosphorylation of tyrosine in the activation loop of the Syk kinase domain [27]. As shown in Fig. 6C, TNF-α increased tyrosine phosphorylation of Syk, while it was inhibited by PI3K inhibitors and NAC. PI3K-siRNA also inhibited TNF-α-induced tyrosine phosphorylation of Syk (Fig. 6D).

3.7. Syk and JNK independently regulate AP-1

Like Syk, JNK was also downstream to ROS and required for AP-1 activation in our previous study [13]. Thus, we explored the relation between Syk and JNK in the signal pathway for TNF-α-induced ET-1 production. Both Syk inhibitors and PI3K inhibitors had no effect on TNF-α-induced phosphorylation of JNK, while NAC inhibited it (Fig. 7A). JNK inhibitor (SP600125, 10 μM) had no effect on TNF-α-induced tyrosine phosphorylation of Syk (Fig. 7B). To further investigate the relation between Syk and MAPKs in TNF-α-induced signal transduction pathway, we examined the activation of MAPKs after TNF-α stimulation in the cells transfected Syk-siRNA or control-siRNA. As shown in Fig. 7C, Syk-siRNA suppressed the Syk protein expression, but did not have an effect on TNF-α-induced JNK, p38 MAPK, or ERK activation.

4. Discussion

ET-1 released from aortic endothelial cells promotes the progression of atherosclerosis [1]. To investigate a possibility of the implication of Syk in the development of atherosclerosis, we explored whether Syk mediates TNF-α-induced ET-1 production in HAECs. TNF-α activated Syk, whereas both Syk inhibitors, Bay 61-3606 and Syk inhibitor 574711, attenuated TNF-α-induced ET-1 production. In addition, specific inhibition of Syk by transfection of Syk-siRNA also downregulated TNF-α-induced ET-1 gene

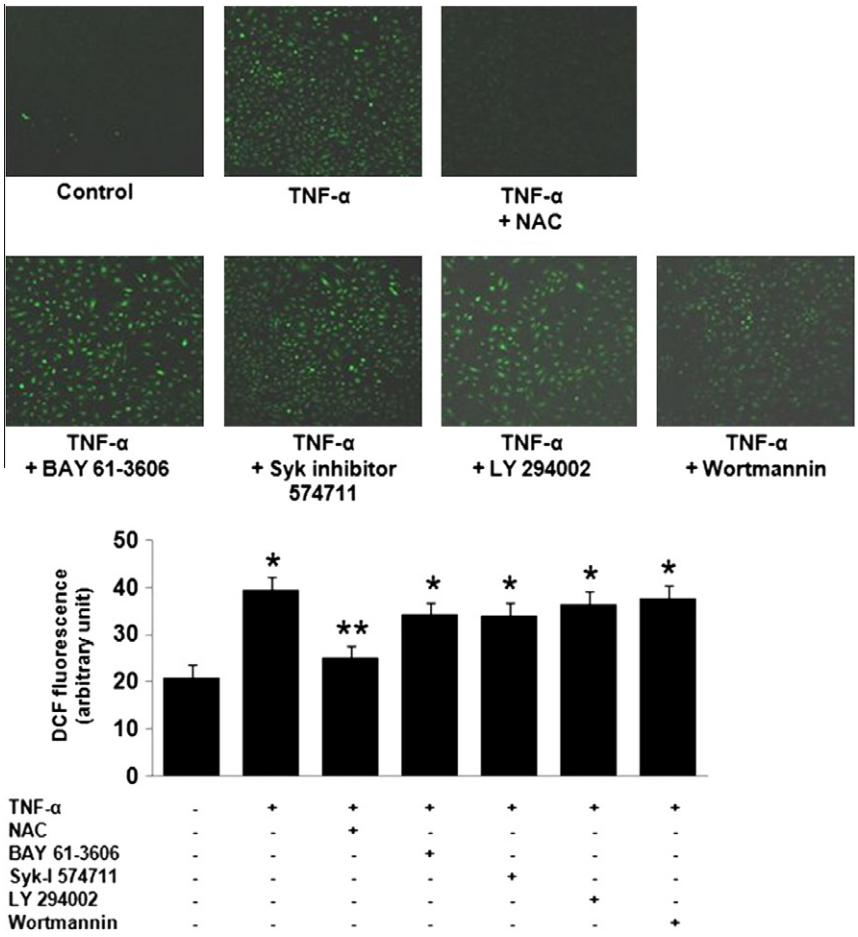

Fig. 5. ROS is upstream to Syk and PI3K. Serum-starved HAECs were treated with/without NAC (5 mM), Syk inhibitors (Bay 61–3606, 1 μM: Syk inhibitor 574711, 1 μM) or PI3K inhibitors (LY 294002, 25 μM: wortmannin, 0.1 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 30 min. Thereafter, the cells were loaded with 5 μM CM-H_2DCF-DA and incubated for 10 min at 37 °C. After removal of the media, the cells were washed and the expression of intracellular ROS was visualized with confocal microscopy. (×200). The bar graph shows relative DCF fluorescent intensities ($^*P < 0.05$ compared with control; $^{**}P < 0.05$ compared with TNF-α; $n = 3$).

expression. These findings together demonstrate that Syk is implicated in TNF-α-induced ET-1 production.

In a previous study of HAECs [13], we demonstrated that TNF-α induces ET-1 gene expression through AP-1 and NF-κB. TNF-α activates all the 3 major MAPKs, including JNK, p38 MAPK and extracellular signal-regulated kinase (ERK). Of the MAPKs, JNK and p38 MAPK, but not ERK, regulate the activations of both AP-1 and NF-κB that were required for TNF-α-induced ET-1 gene expression. The activation of JNK was regulated by upstream ROS. Though p38 MAPK has been shown to be redox-sensitive in several types of cells [28,29], it was activated independently of ROS in HAECs. This was consistent with another our previous study [30], in which darbepoetin alfa that has an antioxidant effect suppressed TNF-α induced JNK activation, but not p38 MAPK activation. The existence of ROS-independent p38 MAPK activation pathway in the downstream of TNF-α signaling was also suggested in other studies [31–33]. On the basis of these findings, in the present study, we further investigated how Syk is involved in the signal pathway for TNF-α-induced ET-1 gene expression. Syk activation was inhibited by NAC while the generation of ROS was not inhibited by Syk inhibitors. Thus, our data showed that Syk is activated downstream to ROS. PI3K also mediated TNF-α-induced ET-1 gene expression. In the present study, Syk inhibitors or Syk-siRNA suppressed TNF-α-induced tyrosine phosphorylation of PI3K, while PI3K inhibitors attenuated Syk activation, suggesting an interaction between Syk and PI3K.

As above, both Syk and JNK were downstream to ROS. With regard to the relation between Syk and JNK, there was a study that Syk regulates TNF-α-induced JNK activation in synoviocytes because Syk inhibitors attenuated TNF-α-induced JNK activation via inhibition of MKK4, the upstream kinase of JNK [22]. However, the signal pathway in HAECs was different from that in synoviocytes. Though both Syk and PI3K also were required for AP-1 activation, either Syk or PI3K was not linked to JNK activation as shown by the findings that Syk-siRNA, inhibitors of Syk or PI3K had no effect on TNF-α-induced JNK activation, and JNK inhibitor also had no effect on TNF-α-induced Syk activation. Thus, Syk and its downstream signal pathway seem to regulate AP-1 independently of JNK. Though the present study did not elucidate how Syk and PI3K regulate AP-1 independently of JNK, there was a study that supports this possibility. In pancreatic cancer cells [34], Akt, which is downstream to PI3K, was shown to directly regulate the transcriptional activity of c-Jun independently of the phosphorylation sites targeted by JNK (Ser63/Ser73).

There was also a study suggesting the regulatory role of Syk in the activation of NF-κB. In hematopoietic cell lines [24], Syk activation by TNF-α resulted in MAPK phosphorylation and NF-κB translocation. On contrast, in HAECs, both Syk inhibitors and PI3K inhibitors could not inhibit either TNF-α-induced p65 nuclear translocation or DNA-binding activity of NF-κB. NAC, an antioxidant, also had no effect on TNF-α-induced p65 nuclear translocation, but it inhibited DNA-binding activity of NF-κB. As shown in

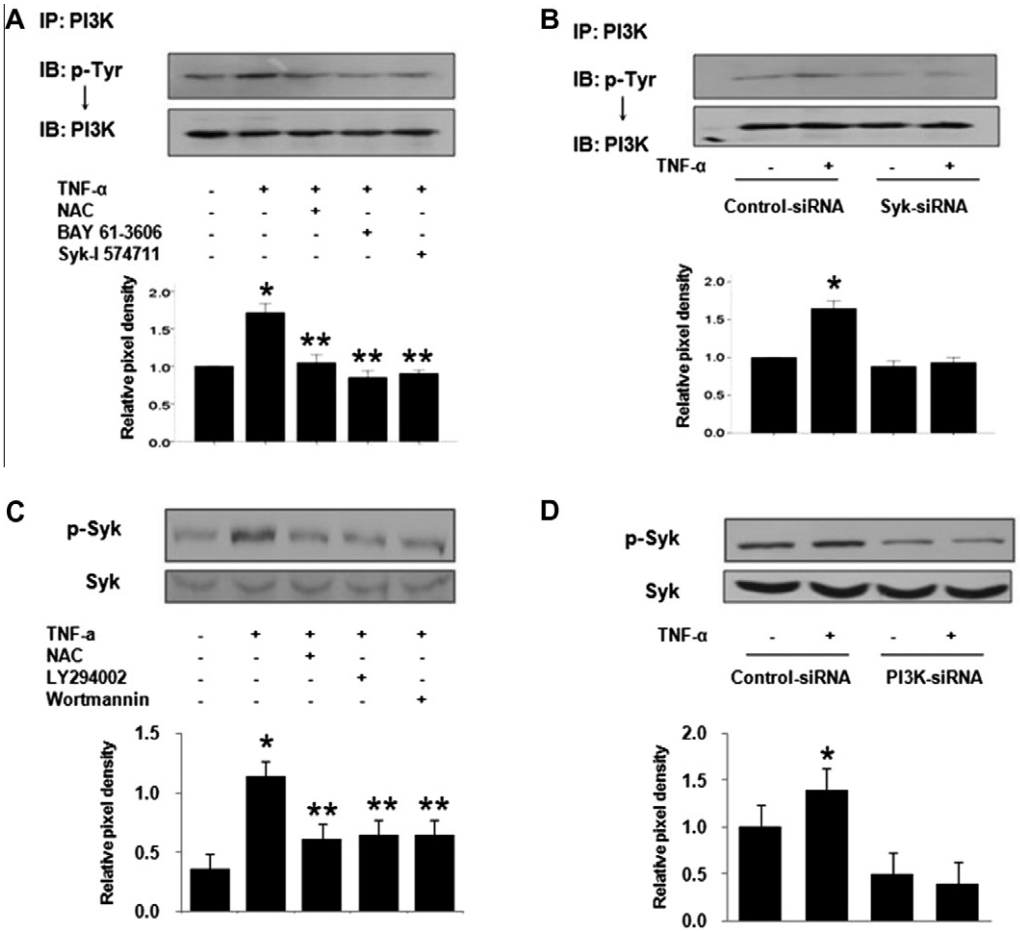

Fig. 6. Syk interacts with PI3K. (A) Effects of NAC or Syk inhibitors on TNF-α-induced PI3K tyrosine phosphorylation. Serum-starved HAECs were treated with/without NAC (5 mM), Bay 61–3606 (1 μM) or Syk inhibitor 574711 (1 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 15 min. The whole cell lysates were immunoprecipitated with anti-PI3K antibody and then subjected to immunoblot analysis using anti-phosphotyrosine antibody. The membranes were stripped and reprobed with anti-PI3K antibody, respectively. The result shown is representative of three independent experiments. ($^{*}P < 0.05$ compared with control; $^{**}P < 0.05$ as compared with cells stimulated with TNF-α). (B) Effects of Syk-siRNA on TNF-α-induced PI3K tyrosine phosphorylation. Cells were transfected with control siRNA or Syk-siRNA and then treated with TNF-α (1 ng/ml) for 15 min. The whole cell lysates were immunoprecipitated with anti-PI3K antibody and then subjected to immunoblot analysis using anti-phosphotyrosine antibody. The membranes were stripped and reprobed with anti-PI3K antibody, respectively. The results shown are representative of three independent experiments. ($^{*}P < 0.05$ compared with control). (C) Effects of NAC or PI3K inhibitors on TNF-α-induced Syk activation. Serum-starved HAECs were treated with/without NAC (5 mM), LY 294002 (25 μM) or wortmannin (0.1 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 10 min. Whole-cell lysates were immunoblotted with an anti-phospho-Syk antibody. Thereafter, the membranes were stripped and reprobed with an anti-Syk antibody. A representative blot from one of four independent experiments is shown. The bar graph shows the relative densities of phospho-Syk/Syk bands ($^{*}P < 0.05$ compared with control; $^{**}P < 0.05$ as compared with cells stimulated with TNF-α). (D) Effects of PI3K-siRNA on TNF-α-induced Syk activation. Cells were transfected with control siRNA or PI3K-siRNA and then treated with TNF-α (1 ng/ml) for 15 min. Phosphorylation of Syk was measured as in C. The results shown are representative of five independent experiments. The bar graph shows the relative densities of phospho-Syk/Syk bands ($^{*}P < 0.05$ compared with control).

our previous study [13], NAC inhibits ROS-dependent activation of JNK as well as ROS-dependent Syk. Inhibition of JNK by NAC results in inhibition of Ser276 phosphorylation of p65 that is required for DNA-binding of NF-κB. This also supports that Syk and JNK are not in a vertical line in the pathway for TNF-α-induced ET-1 gene expression in HAECs.

Taken together the findings of the present and our previous studies, TNF-α-induced intracellular signal pathways leading to ET-1 gene expression in HAECs may be summarized as shown in Fig. 8. TNF-α-induced ET-1 gene expression occurs through JNK and p38 MAPK, both of which regulate AP-1 and NF-κB. Both Syk and JNK are dependent on ROS while p38 MAPK is not. With regard to NF-κB activation, JNK and p38 MAPK has no effect on either IκBα degradation or nuclear translocation of p65, but increases p65 phosphorylation on serine 276 and DNA binding activity of NF-κB. Syk interacts with PI3K and both regulates AP-1 activation independently of JNK.

Recently, Syk has emerged as a new promising therapeutic target for chronic inflammatory diseases including allergic rhinitis [18], rheumatoid arthritis [19] and lupus nephritis [20]. Phase 2 clinical trials of fostamatinib, an oral form of Syk inhibitor, demonstrated considerable beneficial effects on rheumatoid arthritis [19] and immune thrombocytopenic purpura [35]. Syk could also be a potential therapeutic target for atherosclerosis. In a recent study [25], atherosclerotic lesions of human carotid arterial tissue as well as mice aortic root were shown to contain more phosphorylated Syk than healthy control tissues. In addition, the Syk inhibitor, fostamatinib, dose-dependently attenuated atherosclerotic lesion in low-density lipoprotein receptor-deficient mice consuming a high-cholesterol diet. In another study [36], Syk was shown to mediate minimally oxidized low-density lipoprotein-induced stimulation of macropinocytosis resulting in lipid accumulation in macrophages, which is an early and critical step in the initiation and progression of

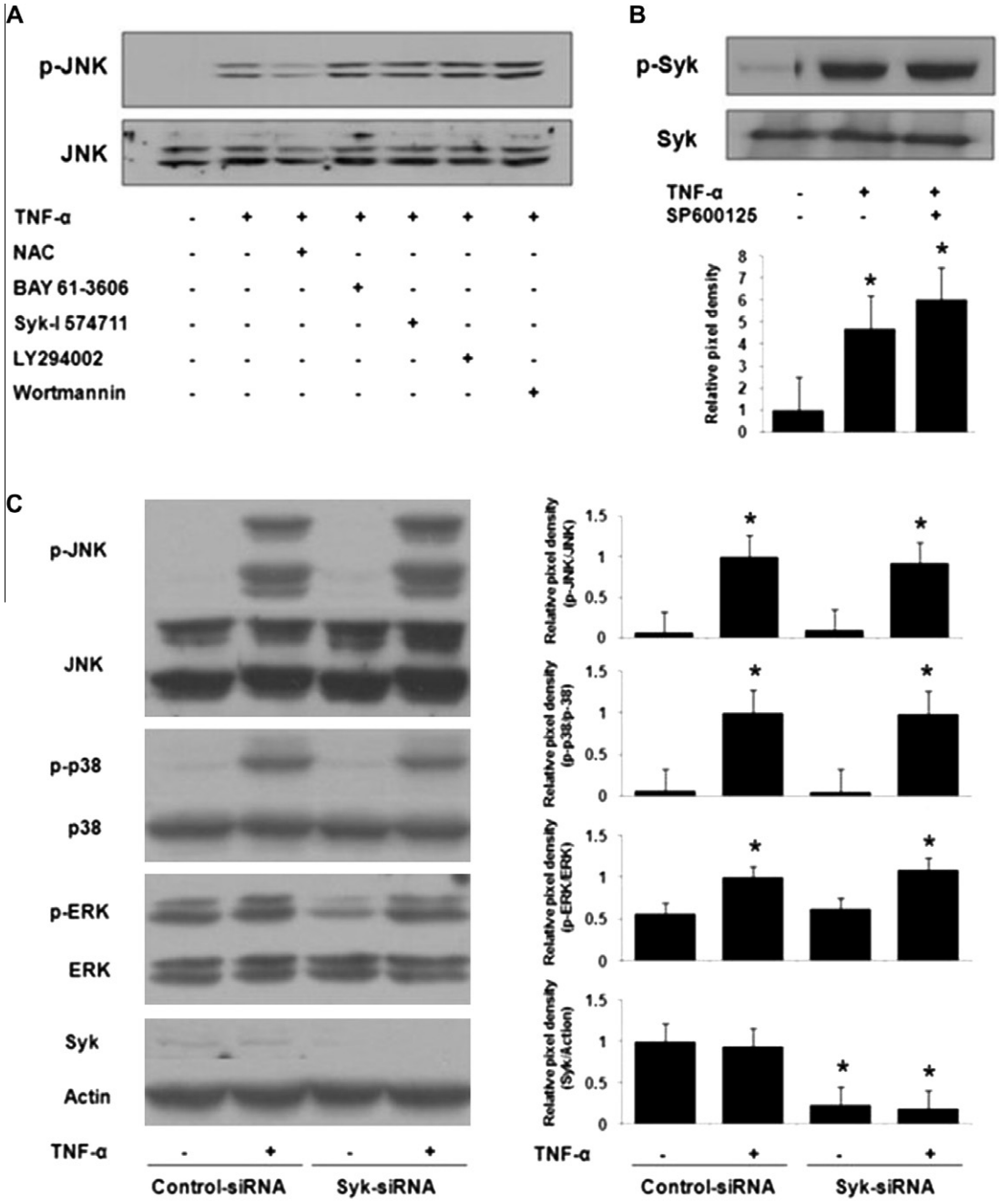

Fig. 7. Syk and JNK independently regulate AP-1. (A) Effects of NAC, Syk inhibitors or PI3K inhibitors on TNF-α-induced JNK phosphorylation. Serum-starved HAECs were treated with/without NAC (5 mM), Bay 61–3606 (1 μM) or Syk inhibitor 574711 (1 μM), LY 294002 (25 μM) or wortmannin (0.1 μM) for 30 min and then incubated with TNF-α for 15 min. The whole cell lysates were analyzed by western blotting with anti-phospho-JNK antibody. The membranes were stripped and reprobed with anti-JNK antibody, respectively. The result shown is representative of three independent experiments. (B) Effect of JNK inhibitor on TNF-α-induced tyrosine-phosphorylation of Syk. Serum-starved HAECs were treated with/without SP6001125 (10 μM) for 30 min and then incubated with TNF-α (1 ng/ml) for 10 min. Whole-cell lysates were immunoblotted with an anti-phospho-Syk antibody. Thereafter, the membranes were stripped and reprobed with an anti-Syk antibody. A representative blot of three independent experiments is shown. The bar graph shows the relative densities of phospho-Syk/Syk bands (*$P < 0.05$ compared with control). (C) Effects of Syk-siRNA on TNF-α-induced MAPK activations. Cells were transfected with control siRNA or Syk-siRNA and then treated with TNF-α (1 ng/ml) for 20 min. The whole cell lysates were immunoblotted with an anti-phospho-JNK, anti-phospho-p38 MAPK, anti-phospho-ERK, or anti-Syk antibodies. Thereafter, the membranes were stripped and reprobed with anti-JNK, anti-p38 MAPK, anti-ERK, or anti-actin antibodies. The results shown are representative of four independent experiments. The bar graphs show the relative densities of phospho-JNK/JNK, phospho-p38 MAPK/p38 MAPK, phospho-ERK/ERK, Syk/actin bands (*$P < 0.05$ compared with control).

atherosclerosis. Because ET-1 is pro-atherogenic [7], the critical role of Syk in TNF-α-induced ET-1 gene expression shown in the present study also supports the possibility that Syk could be implicated in atherosclerosis.

In summary, TNF-α-induced ROS activated Syk and PI3K that were required for the activation of AP-1 and subsequent ET-1 gene transcription. Through the regulation of ET-1 production, Syk could be implicated in atherosclerosis.

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None.

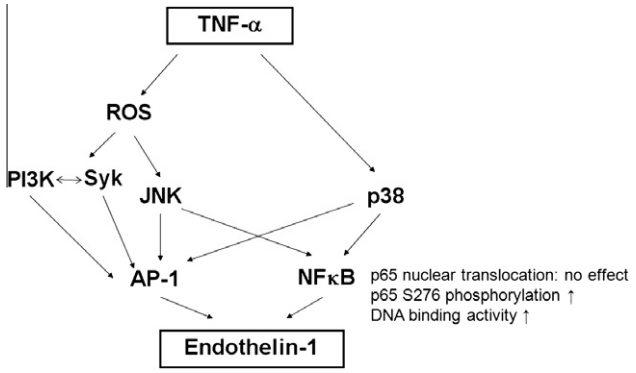

Fig. 8. A schematic diagram demonstrating TNF-α-induced intracellular signal pathways leading to ET-1 gene expression in HAECs and the role of Syk, based on the findings of the present study and our previous studies. TNF-α-induced ET-1 gene expression occurs through JNK and p38 MAPK, both of which regulate AP-1 and NF-κB. Both Syk and JNK are dependent on ROS while p38 MAPK is not. With regard to NF-κB activation, JNK and p38 MAPK has no effect on either IκBα degradation or nuclear translocation of p65, but increases p65 phosphorylation on serine 276 and DNA binding activity of NF-κB. Syk interact PI3K and both regulates AP-1 activation independently of JNK.

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