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RXR agonists inhibit high glucose-induced upregulation of inflammation by suppressing activation of the NADPH oxidase-nuclear factor-κB pathway in human endothelial cells

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ABSTRACT. An inflammatory response induced by high glucose is a cause of endothelial dysfunction in diabetes and is an important contributing link to atherosclerosis. Diabetes is an independent risk factor of atherosclerosis and activation of retinoid X receptor (RXR) has been shown to exert anti-atherogenic effects. In the present study, we examined the effects of the RXR ligands 9-*cis*-retinoic acid (9-*cis*-RA) and SR11237 on high glucose-induced inflammation in human umbilical endothelial vein endothelial cells (HUVECs) and explored the potential mechanism.

Our results showed that the inflammation induced by high-glucose in HUVECs was mainly mediated by the activation of nuclear factor-B (NF-κB). High glucose-induced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were in comparison, significantly decreased by treatment with RXR. The effect of RXR agonists was mainly due to the inhibition of NF-κB activation. Using pharmacological inhibitors and siRNA, we confirmed that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was an upstream activator of NF-κB. Furthermore, RXR agonists significantly inhibited high glucose-induced activation of NADPH oxidase and significantly decreased the production of reactive oxygen species (ROS). To explore whether the rapid inhibitory effects of RXR agonists were in fact mediated by RXR, we examined the effect of RXR downregulation by RXR siRNA. Our results showed that RXR siRNA largely abrogated the effects of RXR agonists, suggesting the requirement of RXR expression. Therefore, we have shown that RXR is involved in the regulation of NADPH oxidase- NF-κB signal pathway, as the RXR ligands antagonized the inflammatory response in HUVECs induced by high glucose.

Key words: Retinoid X receptor; Endothelial cells; High glucose; NF-κB; NADPH oxidase; Inflammation

INTRODUCTION

Endothelial dysfunction has long been recognized as an initiating factor in the development of atherosclerosis and has been linked to elevated glucose level and other cardiovascular risk factors (Xiao et al., 2011). With the accumulation of lipoproteins within the arterial subendothelium, a critical multi-process step ensues including the recruitment of monocytes, which trigger the activation of endothelial cells and the subsequent induction of pro-inflammatory genes including those coding for adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Fotis et al., 2012). It has been established that one of the key transcriptional regulators, NF-κB and its downstream signaling pathway, may play an important role in high glucose-induced endothelial dysfunction (Chen et al., 2009; Li et al., 2011). The Rel/NF-κB family of proteins consists of homo- or hetero-dimers of p49, p50, p52, p65, Rel B, and C rel subunits. The p50/p65 complex is the most abundant heterodimer of NF-κB in cultured endothelial cells (Li et al., 2012). As a consequence of NF-κB activation, the p50/p65 heterodimeric complex translocates into the nucleus and binds specific DNA recognition sequences (Yamamoto and Gaynor, 2004). We previously demonstrated that nicotinamide adenine dinucleotide phosphate (NADPH) oxidases were the major sources of reactive oxygen species (ROS) production in human endothelial cells (Chai et al., 2008). In addition, NADPH oxidases are also involved in the pathophysiology of atherosclerosis (Sorescu et al., 2002). Recent study has shown that ROS produced by NADPH oxidase in activated endothelial cells, participated in the activation of NF-κB pathway (Lin et al., 2005).

Retinoid X receptor (RXR) is a unique member of the nuclear hormone receptor superfamily as it forms heterodimers with many nuclear receptors such as peroxisome

proliferator-activated receptor (PPAR) and liver X receptor (LXR), which play critical roles in regulating vascular functions and lipid metabolism (Kim et al., 2009). 9-*cis*-RA is a natural ligand for RXR and many synthetic compounds with selectivity for RXR have been developed (Dawson and Zhang, 2002). RXR agonists have been shown to inhibit the progression of atherosclerosis in two experimental mouse models, one with mixed dyslipidemia and the second with blood flow cessation (Haraguchi et al., 2006; Lalloyer et al., 2006). *In vitro* studies revealed that RXR agonists decreased cytokines expression induced by inflammation (Xu et al., 2006; Kim et al., 2007), however the underlying mechanism remains poorly understood.

In this study, we investigated the effects of RXR ligands on inflammation induced by high glucose in human endothelial cells and explored the underlying principles of regulation. Out results demonstrated that NADPH oxidase-NF-κB signaling pathway is involved in the inflammation response induced by high glucose in human umbilical endothelial vein endothelial cells (HUVECs). Furthermore, treatment of HUVECs with RXR ligands attenuated the effects of high glucose by inhibiting activation of NADPH oxidase and NF-κB. The inhibitory effects of RXR ligands on inflammatory signaling and the NADPH oxidase-NF-κB pathway, demonstrated the requirement of RXR in these processes.

MATERIAL AND METHODS

Isolation and culture of human endothelial cells

HUVECs were isolated from umbilical cords according to the Declaration of Helsinki as previously described (Chai et al., 2008). The purity of HUVECs in culture was higher than 95% and only passages 1 to 3 were used in the study to avoid age-dependent cellular modifications. Glucose (Sigma, USA) solution was added up to 20 mM (final concentration) for high glucose treatment in our experiments.

Reverse transcription and quantitative real-time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen, USA). 1.5 microgram of total RNA was reverse-transcribed by AMV transcriptase (Roche, Germany) in a total volume of 25 μL. Using 2.0 μL of the product as a template, we performed quantitative real-time PCR using the following primers: ICAM-1: sense, 5'-TGG TAG CAG CCG CAG TCG TA-3' and antisense, 5'-CTC CTT CCT CTT GGC TTA GT-3'; VCAM-1 sense, 5'-CCC TTG ACC GGC TGG AGA TT-3' and antisense, 5'-CTG GGG GCA ACA TTG ACA TAA AGT G-3'; and GAPDH sense, 5'-CGG TAT CGT GGA AGG ACT CAT G-3' and antisense, 5'-TCC TTG GAG GCC ATG TGG GCC AT-3'. Standard curves were performed using a serial dilution of the standard template. Quantitative measurements were evaluated by the △△Ct method. The expression of GAPDH was used as the internal control. The mRNA expression of control group was expressed as 100%. Fold-induction of mRNA expression was calculated.

Transfection of small interfering RNA (siRNA)

To knock down the Nox4 expression in HUVECs, we tested three predesigned siRNA sequences obtained from GenePharma (Shanghai, China). We found that only the sequence tar-

geting exon 2 consistently reduced Nox4 expression in HUVECs, thus this siRNA (sequence: GGA TAC AGC CAT GCC GCC TAA T) was used in the following experiments. Predesigned siRNAs against human RXRα (sequence: GGC GAT ATG GCT GTG TCC CGG C) and controlled scrambled siRNAs (sequence: ATT GGA CCA AGT GGT TCA TAG C) were also synthesized by GenePharma. HUVECs grown to 60-70% confluence were transfected with 12.5-25 nM oligonucleotides by using the Transpass R2 transfection reagent (New England BioLabs, UK) under serum-free conditions according to manufacturer protocol. After transfection, cells were incubated with complete medium for 48 h before assaying target gene inhibition.

Cellular fractionation and immunoblotting

Nuclear and cytoplasmic extractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, USA). To obtain cytoplasmic proteins, HUVECs were lysed in hypotonic lysis buffer, and membrane fractions (pellets) were obtained by ultracentrifugation (100,000 g for 1 h). For immunoblotting, total cellular proteins (50 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Antibodies used were: rabbit monoclonal antibody against β-actin (Lab vision corporation, USA), rabbit polyclonal antibody against ICAM-1, rabbit polyclonal antibody against VCAM-1, rabbit monoclonal antibody against histone H1, rabbit monoclonal antibody against RXRα, and mouse monoclonal antibody against human NF-κB p65 (Santa Cruz, USA).

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from HUVECs were prepared and analyzed by LightShift Chemiluminescent EMSA kit (Pierce, USA) according to manufacturer protocol. Briefly, binding reactions containing equal amounts of protein (10 μg) and 20 fmol of biotin-labeled NF-κB oligonucleotide (probe) were incubated for 20 min in binding buffer (10 mM Tris-Cl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, 1 μg poly (dI·dC) and 2.5% glycerol). Binding reactions were analyzed using 6% native PAGE. After blotting to a nylon membrane, labeled oligonucleotides were detected by chemiluminescence.

Immunofluorescent detection of NF-κB p65

HUVECs were seeded in chamber slides coated with 0.1% polylysin (Sigma-Aldrich, USA) and grown to confluence. After treatments, cells were fixed in 4% paraformaldehyde. After permeabilization with 0.01% Triton X-100 for 15 min at room temperature and blocking with 3% BSA, cells were incubated with mouse monoclonal antibody against human NF-κB p65 (Santa Cruz, USA). The corresponding secondary antibody used was goat anti-mouse FITC-labeled secondary antibody (Santa Cruz, USA). Images were acquired using a laser confocal microscopy (Leica Microsystems, Wetzlar, Germany).

Detection of the production of ROS by flow cytometry

ROS production was measured in 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) (Sigma, USA)-treated HUVECs by flow cytometry using Coulter Epics XL (Beck-

man Counter, USA) as we previously described (Chai et al., 2008).

NADPH oxidase enzymatic activity assay

NADPH oxidase activity was measured using a lucigenin assay. Cells were lysed in a buffer containing protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail Kit, Pierce, USA). Protein lysates were collected and resuspended in TRIS-sucrose buffer, and protein content was measured by the Bradford method (Coomassie Plus, the Better Bradford Assay Kit, Pierce, USA). NADPH oxidase activity was performed in a phosphate buffer (50 mM, pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin as the electron acceptor, and NADPH (100 μM) as the substrate (final volume 1 mL). The reaction was initiated by the addition of 100 μg of protein. Photon emission was measured every 30 s for 30 min in a scintillation counter (Orion II, Berthold Detection System, Germany). Background counts were determined by protein-free incubations and were subtracted from protein readings. Results were expressed in cpm/mg protein/min.

Statistical analysis

Data are reported as means ± SE. Results were compared by One-way factorial ANOVA followed by a post hoc Scheffé's comparison test. A value of $P \leq 0.05$ was considered to be significant.

RESULTS

Induction of inflammation by high glucose requires NF-κB

High glucose is well known to induce the production of adhesion molecules in endothelial cells (Bacun et al., 2010). To determine whether NF-κB activation was involved, HUVECs were cultured in medium containing high glucose (20 mM) in the presence or absence of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) or BAY 11-7082([E]-3-[4-methylphenylsulfonyl]-2-propenenenitrile). Equimolar concentration of mannitol was used in control cells for the osmotic effects of high glucose. Exposure of HUVECs with high glucose for 24 h resulted in increased expression of ICAM-1 and VCAM-1. When HUVECs were pretreated with the indicated concentration of PDTC or BAY 11-7082 for 1 h, high glucose-induced ICAM-1 and VCAM-1 expression were inhibited (Figure 1). Thus, NF-κB activation was involved in high-glucose induced upregulation of adhesion molecules in HUVECs.

Induction of inflammation by high glucose requires NADPH oxidase

Human endothelial cells express all classic NADPH oxidase subunits, including membrane bound Nox4 (Gorlach et al., 2000). We previously demonstrated that Nox4 was the critical component of NADPH oxidase activation in HUVECs. To determine whether NADPH oxidases were involved in inflammation induced by high glucose, we examined the effects of NADPH oxidase inhibitors apocynin (Apo) or diphenyleneiodonium (DPI) on high glucose-induced the expression of ICAM-1 and VCAM-1. Treatment of HUVECs grown in

high-glucose medium with Apo or DPI significantly decreased the production of ICAM-1 and VCAM-1 to a level close to the control value. Furthermore, we transfected HUVECs with siRNA targeted towards Nox4. The expression of ICAM-1 and VCAM-1 induced by high glucose was significantly inhibited in cells transfected with Nox4 targeted siRNA (Figure 2). Real-time PCR and immunoblotting demonstrated that neither transfection procedure itself nor the transfection of HUVECs with a control of nonspecific siRNA (scrambled siRNA) had an effect on ICAM-1 and VCAM-1. In addition to NF-κB, we determined NADPH oxidase was also required in high-glucose induced expression of ICAM-1 and VCAM-1.

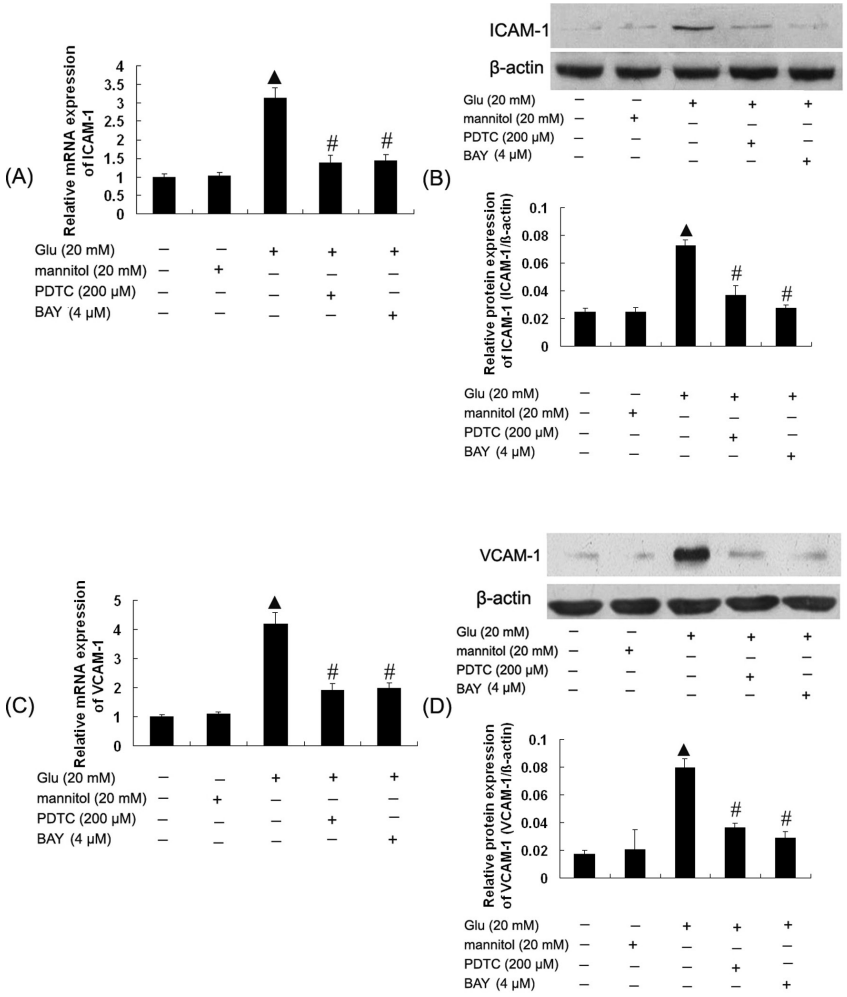

Figure 1. Effects of NF-κB inhibitors on expression of ICAM-1 (**A**-**B**) and VCAM-1 (**C**-**D**). HUVECs were incubated with PDTC (200 μM) and BAY-11-7082 (4 μM) for 1 h and then exposed to high glucose (20 mM) and mannitol (20 mM) for 24 h. Levels of ICAM-1 (**A**) and VCAM-1 (**C**) mRNAs were determined by real-time PCR. The inhibiting effects of NF-κB inhibitors were confirmed at protein level by immunoblotting. Representative immunoblots (N = 4) are shown and the relative ratio over β-actin were determined by densitometric analysis. Data are reported as means ± SE (N = 4). ▲P < 0.05, compared to control (5.5 mM glucose), #P < 0.05, compared to high glucose (20 mM).

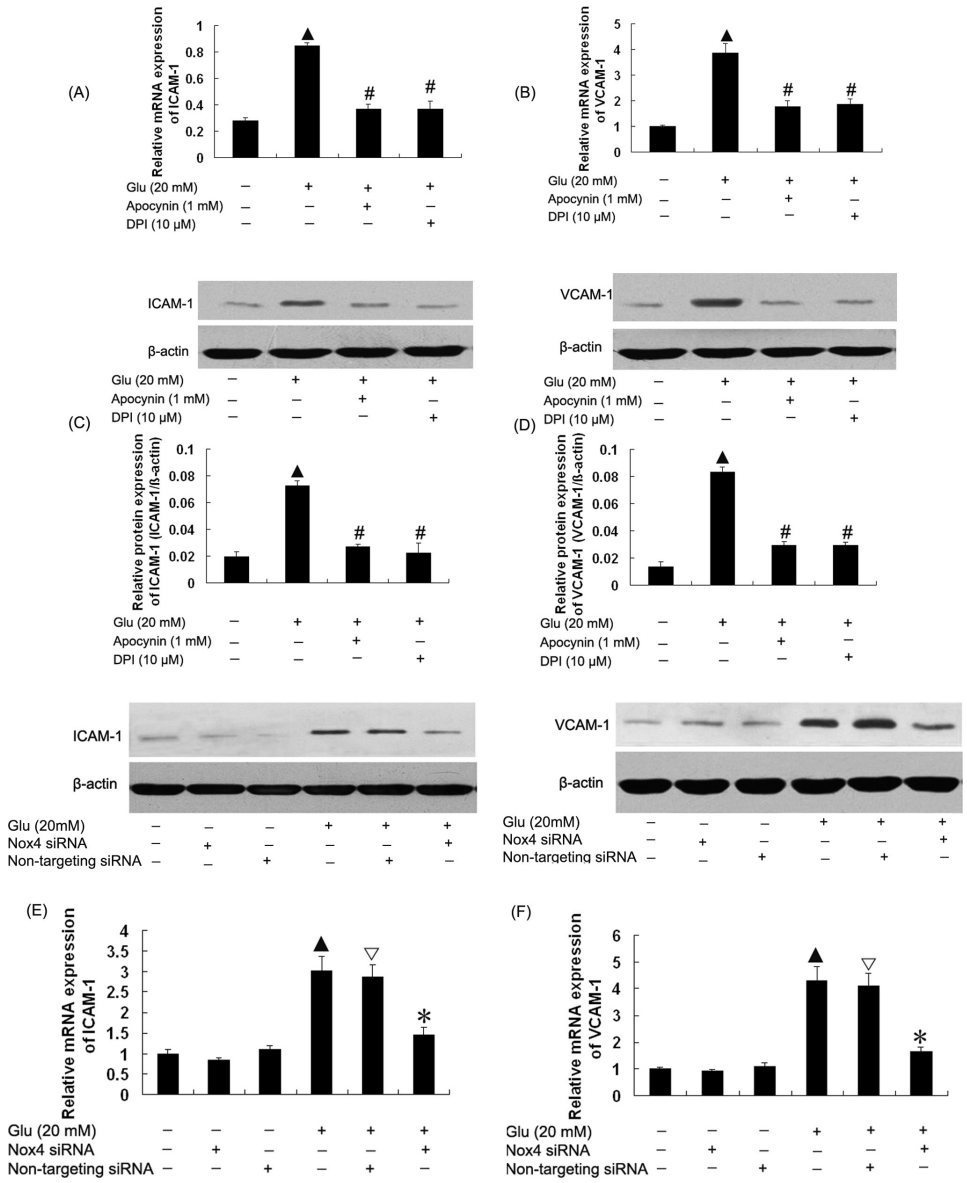

Figure 2. Effects of NADPH oxidase inhibitors on expression of ICAM-1 (A-B) and VCAM-1 (C-D). HUVECs were incubated with Apocynin (1 mM) and DPI (10 μM) for 1 h and then exposed to high glucose (20 mM) and mannitol (20 mM) for 24 h. Levels of ICAM-1 (**A**) and VCAM-1 (**C**) mRNAs were determined by real-time PCR. The inhibiting effects of NF-kB inhibitors on ICAM-1 (**B**) and VCAM-1 (**D**) were confirmed at protein level by immunoblotting, the relative ratio over β-actin were determined by densitometric analysis and the representative immunoblots (N = 4) are shown. **E. F.** Knockdown of Nox4 by siRNA in HUVECs. HUVECs were transfected with scrambled siRNA and Nox4 siRNA. The mRNA levels of ICAM-1 (**E**) and VCAM-1 (**F**) were determined by real-time PCR. Protein levels were examined by immunoblotting. Data are reported as means ± SE (N = 4). ▲P < 0.05, compared to control (5.5 mM glucose); #P < 0.05, compared to 20 mM glucose; ▽P < 0.05, compared to scrambled; *P < 0.05, compared to 20 mM glucose plus scrambled.

High glucose-induced activation of NF-κB requires NADPH oxidase

The data described above demonstrated that NF-κB and NADPH oxidase were required in high glucose-induced inflammation. Using immunoblotting and confocal microscopy analysis, we next examined whether the NADPH oxidase inhibitors and Nox4 siRNA, decreased high glucose-induced activation of NF-κB. For verification of the effects of NADPH oxidase inhibitors and Nox4 siRNA on NF-κB DNA-binding activity, a mobility shift assay (EMSA) was carried out. HUVECs were cultured in medium containing high glucose in the presence or absence of NADPH oxidase inhibitor DPI or Apo. Equimolar concentrations of mannitol were used in control cells for the osmotic effects of glucose. EMSA analysis showed a significant increase in NF-κB DNA-binding activity after 1 h of exposure to high glucose (Figure 3). In contrast, pretreatment of HUVECs grown in high glucose medium with either Apo or DPI decreased the NF-κB DNA-binding activity to a level close to the control value (Figure 3).

HUVECs transfected with Nox4 or scrambled siRNA were exposed to high glucose and we were able to demonstrate the high glucose-induced NF-κB binding activity was also significantly decreased by Nox4 siRNA (Figure 3). To confirm the binding activity obtained from our EMSA results, we prepared nuclear and cytosolic fractions of HUVECs. Immunoblotting of fractions with anti-NF-κB p65 antibody showed that exposure of cells to high glucose resulted in enhanced NF-κB p65 subunit levels in the nuclear fraction, with a corresponding decrease in the cytoplasmic fraction. We concluded that the decrease of NF-κB p65 in the cytoplasm was not due to degradation of cytoplasmic p65 subunit, as NADPH oxidase inhibitors and high glucose p65 nuclear accumulation was abrogated by DPI, Apo treatment, and Nox4 siRNA transfection (Figure 3). Together, these results suggest that high-glucose-induced NF-κB activation requires NADPH oxidase.

RXR agonists inhibit the expression of ICAM-1 and VCAM-1 induced by high glucose

Next, we examined whether RXR ligands 9-*cis*-RA and SR11237 modulated the effects of high glucose on the expressions of ICAM-1 and VCAM-1. HUVECs were pretreated with 9-*cis*-RA or SR11237 in indicated concentrations for 1 h, followed by high glucose treatment for 24 h. Gene expression analysis of ICAM-1 and VCAM-1 as shown in Figure 4, demonstrated that 9-*cis*-RA significantly suppressed the high glucose induced upregulation of ICAM-1 and VCAM-1 in a concentration-dependent manner. Inhibition occurred when cells were treated with 10 nM 9-*cis*-RA, whereas about 45-50% of high glucose-induced expression of ICAM-1 and VCAM-1 was inhibited by 100 nM 9-*cis*-RA. SR11237 at 100 nM also showed an inhibitory effect similar to that of 9-*cis*-RA, suggesting a general effect of RXR agonists. The inhibitory effects of 9-*cis*-RA and SR11237 on ICAM-1 and VCAM-1 at a protein level were confirmed by immunoblotting with similar results. Thus, RXR agonists are able to attenuate high glucose-induced inflammation genes.

RXR agonists antagonize the effect of high glucose on NF-κB activation

NF-κB was found to regulate the expression of ICAM-1 and VCAM-1 induced by high

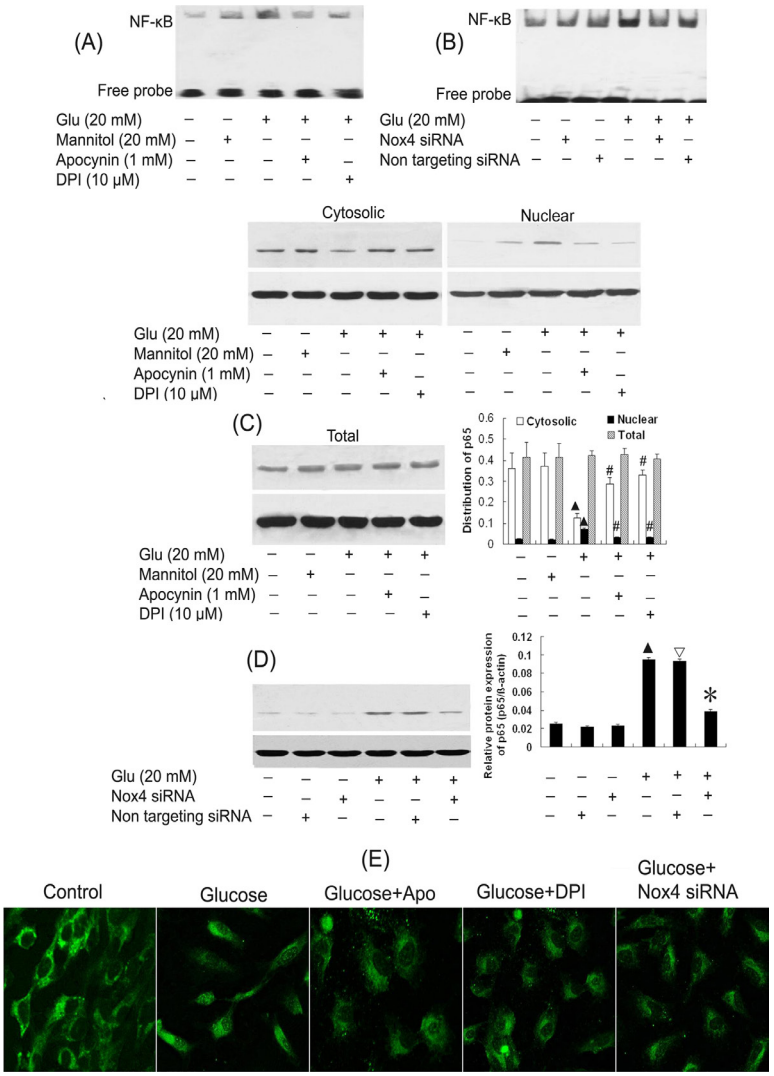

Figure 3. Regulation of NF-κB activation by NADPH oxidase inhibitors and Nox4 siRNA. (A-B) Inhibiton of high glucose-induced NF-κB activation. HUVECs treated (**A**) with the indicated concentrations of Apo and DPI or transfected (**B**) with specific siRNA for Nox4 were exposed to high glucose (20 mM) and mannitol (20 mM) for 1 h. The NF-**κ**B DNA-binding activity was determined by EMSA. A representative EMSA result (N = 3) is shown. **C.** Effects of high glucose and NADPH oxidase inhibitors on p65 subcellular localization. HUVECs were pretreated with indicated concentrations of Apo and DPI for 1 h, then exposed to high glucose for 1 h. Nuclear, cytosolic, and total extracts were prepared and analyzed by immunoblotting. A presentive Western blot (N = 3) is shown. The relative ration of p65 over β-actin was determined by densitometric analysis. **D.** Knockdown of Nox4 by siRNA in HUVECs. HUVECs were transfected with Nox4 siRNA and scrambled siRNA. The cytosolic p65 levels were determined by immunoblotting and normalized to those of β-actin. Data are reported as means ± SE (N = 3). $^{\blacktriangle}P < 0.05$, compared to control (5.5 mM glucose); $^{\#}P < 0.05$, compared to 20 mM glucose; $^{\triangledown}P < 0.05$, compared to scrambled; $^{*}P < 0.05$, compared to 20 mM glucose plus scrambled. **E.** p65 immunostaining in HUVECs. HUVECs transfected with Nox siRNA or pretreated with Apo (1 mM) and DPI (10 μM) were exposed to high glucose for 1 h. Cells were immnostained by anti-p65 antibody and immunofluorescence was detected by confocal microscopy.

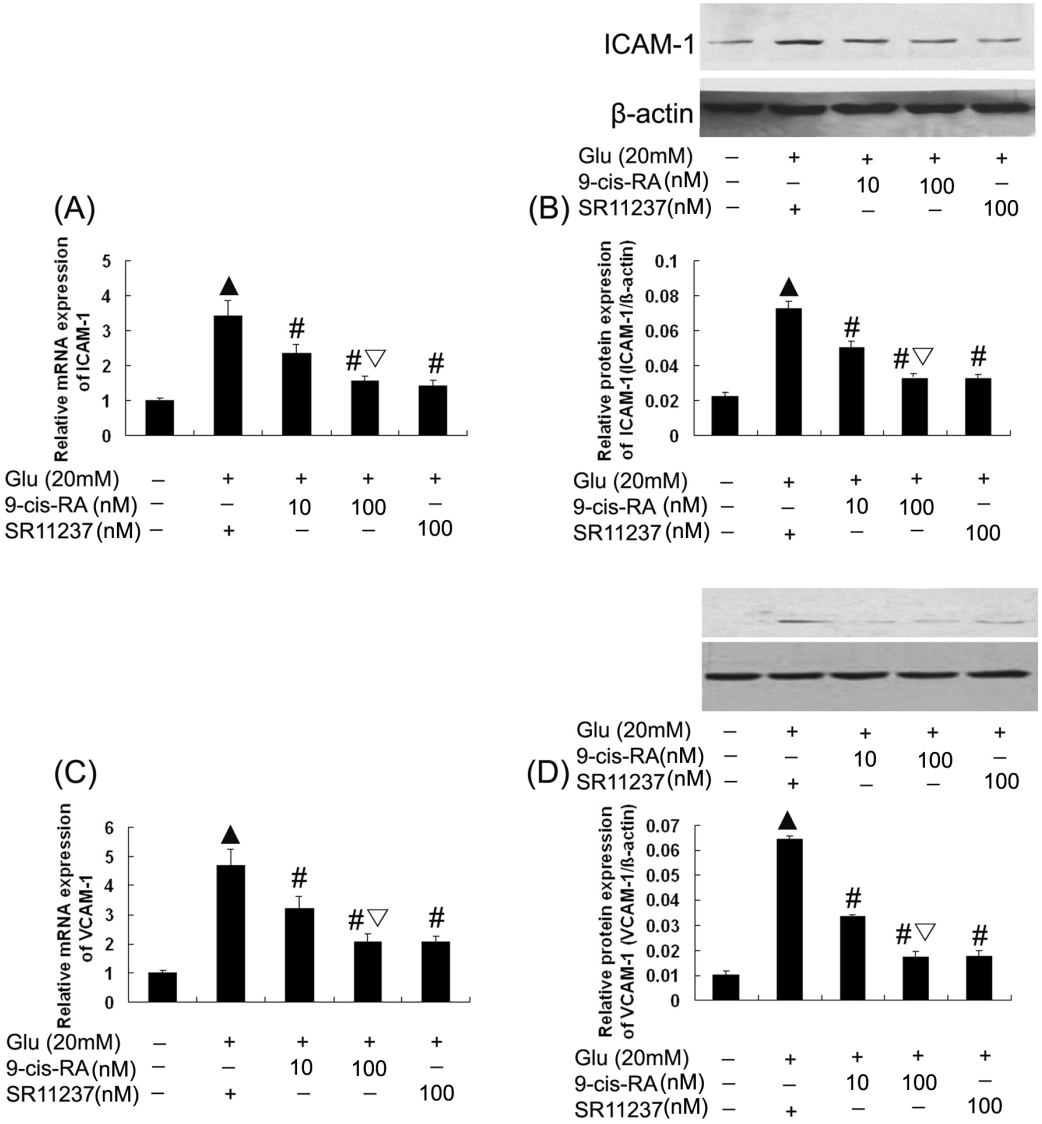

Figure 4. Effects of 9-*cis*-RA and SR11237 on the induction of ICAM-1 and VCAM-1. Inhibition of high glucose-induced (**A**) ICAM-1 and (**C**) VCAM-1 mRNA levels by 9-*cis*-RA and SR11237. HUVECs were treated with indicated concentration of 9-*cis*-RA and SR11237 for 1 h and then high glucose for 24 h. Levels of ICAM-1 and VCAM-1 mRNAs were determined by real-time PCR. The inhibiting effects of 9-*cis*-RA and SR11237 on (**B**) ICAM-1 and (**D**) VCAM-1 were confirmed at protein level by immunoblotting. Representative immunoblots (N = 3) are shown. Data are reported as means ± SE (N = 3). ▲P < 0.05, compared to control (5.5 mM glucose); #P < 0.05, compared to 20 mM glucose; ▽P < 0.05 compared to 20 mM glucose plus 10 nM 9-*cis*-RA.

glucose. To determine whether RXR agonists could suppress high glucose-induced activation of NF-κB, HUVECs were incubated with 9-*cis*-RA or SR11237 for 1 h, followed by high glucose treatment for 1 h. Analysis of NF-κB activation by EMSA revealed that high glucose-induced

NF-κB DNA binding activity was inhibited by 9-*cis*-RA in a concentration-dependent manner and that SR11237 at 100 nM showed an inhibitory effect similar to that of 9-*cis*-RA (Figure 5). Cellular fraction immunoblotting assays showed that high glucose-induced NF-κB subunit p65 nuclear accumulation was inhibited by about 48% by 100 nM 9-*cis*-RA. The degree of inhibition of NF-κB p65 nuclear translocation by 100 nM SR11237 was comparable to that of 100 nM 9-*cis*-RA. The reduction of NF-κB p65 in the cytoplasm induced by high glucose was reversed by 100 nM SR11237 and 100 nM 9-*cis*-RA. We also determined that the RXR agonists and high glucose treatment had no effect on total NF-κB p65 protein level (Figure 5). These results suggest that RXR ligands can antagonize the effect of high glucose on NF-κB activation.

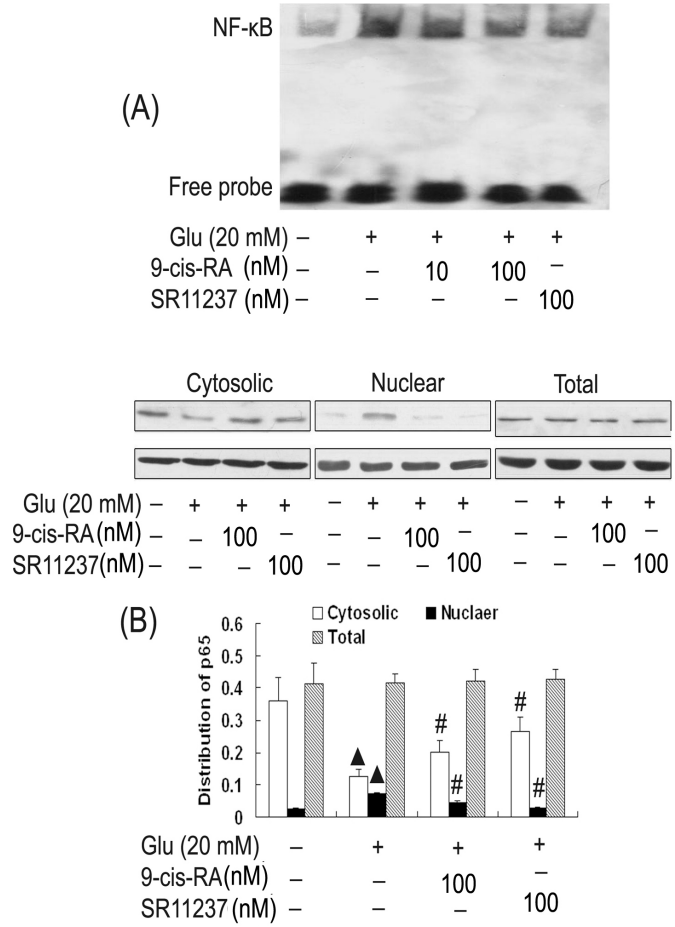

Figure 5. Effect of RXR agonist on NF-κB activation. Inhibiton of high glucose-induced NF-κB activation. HUVECs treated (**A**) with the indicated concentrations of 9-*cis*-RA and SR11237 were exposed to high glucose (20 mM) for 1 h. The NF-κB DNA-binding activity was determined by EMSA. A representative EMSA result (N = 3) is shown. **B.** Effects of high glucose and RXR agonists on p65 subcellular localization. HUVECs were pretreated with indicated concentrations of 9-*cis*-RA and SR11237 for 1 h, then exposed to high glucose for 1 h. Nuclear, cytosolic, and total extracts were prepared and analyzed by immunoblotting. A presentive Western blot (N = 3) is shown. The relative ration of p65 over b-actin was determined by densitometric analysis. Data are reported as means ± SE (N = 3). $^{\blacktriangle}P < 0.05$, compared to control (5.5 mM glucose); $^{\#}P < 0.05$, compared to 20 mM glucose.

RXR agonists reduce high glucose-induced activation of NADPH oxidase

There are several potential sources of superoxide in endothelial cells, including the mitochondrial electron transport chain, xanthine oxidase, nitric oxide synthases, and NADPH oxidase (Ray and Shah, 2005). Using pharmacological inhibitors, we confirmed that only the NADPH oxidase inhibitor, DPI, could reduce high glucose-induced ROS production in endothelial cells. Inhibitors of other sources of ROS, such as allopurinol (xanthine oxidase inhibitor), NG-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor) and rotenone (mitochondrial electron transport chain oxidase inhibitor) had no effects on ROS production (data not shown). Thus, NADPH oxidase plays a crucial role in high glucose-stimulated production of superoxide in human endothelial cells. As previously described, ROS plays a pivotal role in mediating the response of the endothelium to high glucose and the augmented intracellular ROS production can regulate redox-sensitive genes such as adhesion molecules (Gutiérrez et al., 2007). The above observation showed that RXR ligands and NADPH oxidase inhibitors exerted similar inhibitory effects on high glucose-induced inflammation and NF-κB activation, suggesting that RXR agonists might interfere with NADPH oxidase activation. We first examined the effect of high glucose on ROS production by flow cytometry analysis as shown in Figure 6. We demonstrated that exposure of HUVECs to high glucose for 30 min or longer (up to 8 h) resulted in enhanced fluorescence intensity correlating to ROS production. When HUVECs were exposed to 9-*cis*-RA, high glucose-induced production of superoxide was rapidly inhibited in a concentration-dependent manner. Furthermore, treatment with SR11237 at 100 nM also showed an inhibitory effect similar to that of 9-*cis*-RA (Figure 6B). Consistent with these results, both 9-*cis*-RA and SR11237 blunted high-glucose-induced NADPH oxidase activity (Figure 6). Our group has previously demonstrated that RXRα could be detected in both nuclear and cytosolic fractions of endothelial cells (Chai et al., 2008). Recent studies have also revealed the existence of alternative functions of RXR in the cytoplasm (Cao et al., 2004). To investigate whether the inhibitory effects of 9-*cis*-RA was mediated by RXR, HUVECs were transfected with RXR specific siRNAs. The inhibition of high glucose-induced ROS production by 9-*cis*-RA was largely impaired by RXR siRNA, whereas transfection of cells with nonspecific siRNA (scrambled siRNA) did not have any effect (Figure 6). The inhibitory effect of 9-*cis*-RA on high glucose-induced NADPH oxidase activity was also significantly attenuated when HUVECs were transfected with RXRα siRNA (Figure 6). These results demonstrate that RXR mediates NADPH oxidase activation through ligand binding of 9-*cis*-RA.

DISCUSSION

Accelerated atherosclerosis is characteristic for patients with diabetes mellitus and can result in high plasma glucose. This in turn, can induce increased recruitment of plasma monocytes in part by up regulating expression of adhesion molecules in endothelial cells, such as ICAM-1 and VCAM-1. This mechanism represents one of the earliest pathological changes in immune and inflammatory diseases, such as atherosclerosis (Xiao et al., 2011). The present study demonstrated that exposure of HUVECs to high glucose (20 mM) induced the expression of ICAM-1 and VCAM-1, which we confirmed with the use of pharmacological inhibitors, was dependent on NF-κB activation. In addition, NADPH oxidase inhibitors significantly inhibited the effect of high glucose on the expression of ICAM-1 and VCAM-1, which suggested that NADPH oxidase contributed to high glucose-induced expression of adhesion molecules.

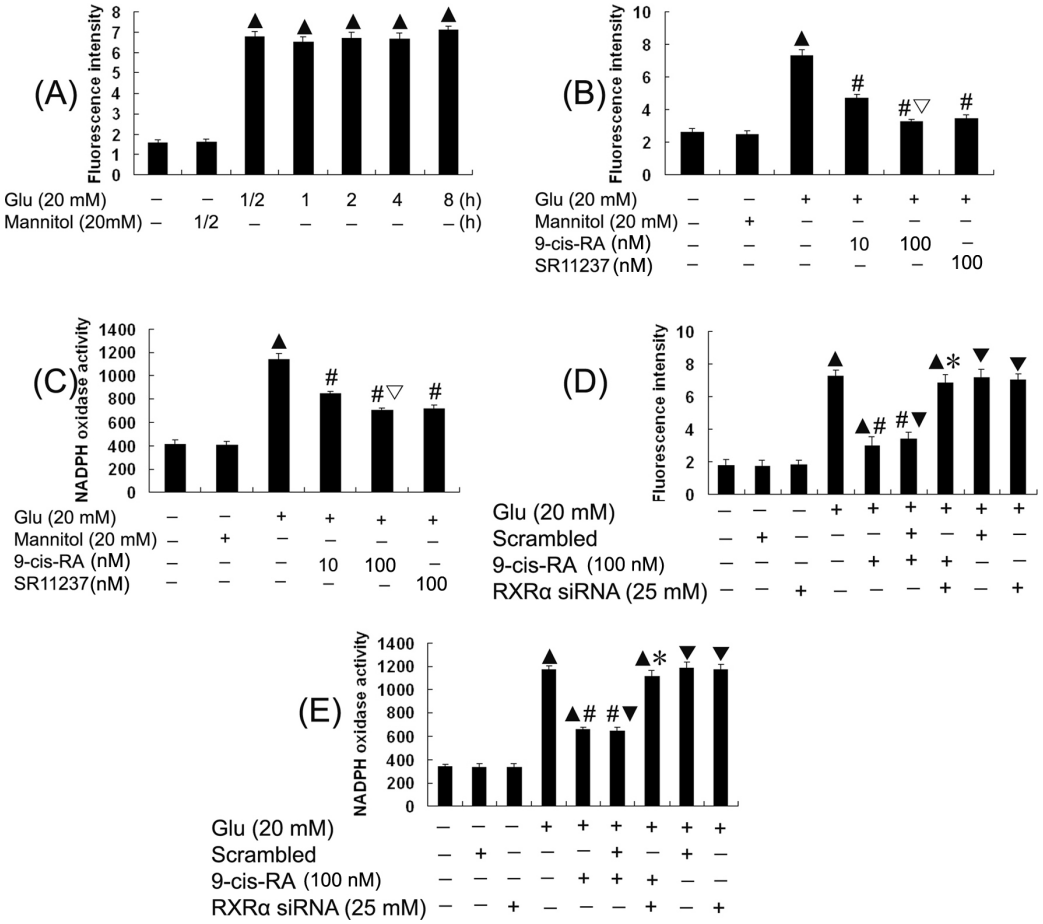

Figure 6. Effect of RXR agonists on NADPH oxidase activation. **A.** Flow cytometry analysis of the effects of high glucose on ROS production. **B.** Inhibiton of high glucose-induced ROS production. **C.** NADPH oxidase activity by 9-*cis*-RA and SR11237. ROS production and NADPH oxidase activity were determined using flow cytometry analysis and lucigenin assay respectively. HUVECs treated with the indicated concentrations of 9-*cis*-RA and SR11237 for 1 h were exposed to high glucose (20 mM) for 30 min. **D. E.** Involvment of RXRα in the inhibition of high glucose-induced ROS production and NADPH oxidase activation by 9-*cis*-RA. HUVECs were transfected with scrambled siRNA or RXRa siRNA (25 mM) were treated with 9-*cis*-RA (100 nM) and then exposed to high glucose for 30 min. ROS production were determined using flow cytometry analysis, NADPH oxidase activity were determined using lucigenin assay. Values are means ± SE (N = 4). $^{\blacktriangle}P < 0.05$, compared to control (5.5 mM glucose); $^{\#}P < 0.05$, compared to 20 mM glucose; $^{\triangledown}P < 0.05$, compared to 20 mM glucose plus 10 nM 9-*cis*-RA; $^{\blacktriangledown}P < 0.05$, compared to 5.5 mM glucose plus scramble; $^{*}P < 0.05$, compared to 20 mM glucose plus 9-*cis*-RA (100 nM) and scrambled.

Nox4, being an important component of the NADPH oxidase family, contributed to the increased NADPH oxidase activity induced by high glucose. This was determined using Nox4 siRNA, which revealed a crucial role in the regulation of high glucose-induced activation of NF-κB and adhesion molecules expression. Interestingly, we report here that RXR ligands could down-regulate adhesion molecules (ICAM-1 and VCAM-1) expression and blunt

activation of NF-κB in human endothelial cells. These findings have considerable implications for preventing endothelial damage in the diabetes mellitus setting.

Inflammation contributes to various stages of atherogenesis (Glass and Witztum, 2001). The process of inflammation is defined as an infiltration of blood leukocytes in to tissues with the aid of chemokines and adhesion molecules. Previous evidence indicates that ROS and NF-κB play an important role in this process (Krötz et al., 2001). In endothelial cells, NADPH oxidase is the major sources of ROS production, which is generated in response to a variety of stimuli required for intracellular signaling in endothelial cell (Maloney et al., 2009; Masai et al., 2010). ROS can regulate intracellular kinase activities through reversible inactivation of phosphatases and can activate transcription factors including NF-κB, which in turn leads to expression changes of numerous downstream genes (Janssen-Heininger et al., 2000). Our results provided evidence that high glucose-induced activation of NADPH oxidase resulted in the production of ROS, which acted as intracellular signal to activate NF-κB pathway. This signaling may serve as a therapeutic target for preventing inflammation induced by high glucose.

Studies over the past decade have identified the key roles for RXR in lipid and glucose metabolism, energy homeostasis, and inflammation control (Szanto et al., 2004; Haraguchi et al., 2006). Retinoids act as potent anti-inflammation agents that exert beneficial responses in the cardiovascular system (Lalloyer et al., 2006; Xu et al., 2006). RXR expressed in endothelial cells and macrophages, exerts an anti-inflammatory effect and is implicated in the progression of arteriosclerosis (Szanto et al., 2004). However, the mechanism in which RXR ligands exert these beneficial effects in vascular endothelial cells remains largely unclear. Our investigation on the effect of 9-*cis*-RA on high glucose-induced inflammation revealed that RXR ligands might exert their vascular protective effects by antagonizing the expression of adhesion molecules and activation of NF-κB induced by high glucose. Interestingly, 9-*cis*-RA decreased the high glucose-induced expression of ICAM-1 and VCAM-1 in endothelial cells by inhibiting the activation of NADPH oxidase. Our finding is consistent with previous observations that the fatty acid DHA, acting as a RXR ligand, inhibits growth and induces apoptosis by inhibiting NF-κB activity and suppressing cytokine production in macrophages (Narayanan et al., 2005; Weldon et al., 2007). DHA was also shown to suppress ROS production in endothelial cells by inhibiting NADPH oxidase activity (Massaro et al., 2006). In line with this, we have shown that the natural RXR ligand 9-*cis*-RA inhibited activation of the NADPH oxidase-NF-κB pathway, which attenuated inflammatory damage to endothelial cells. It could be extrapolated that this may protect against atherogenesis in the setting of high glucose as seen for example in diabetic mellitus. Although 9-*cis*-RA does not binds only to RXR but also to retinoic acid receptor, we showed here that a RXR-specific ligand, SR11237, also exhibited similar inhibitory effects, suggesting that the blunting effects of 9-*cis*-RA are mediated by a RXR specific pathway.

RXR is generally considered to reside in the nucleus and to exert its transcriptional regulation either as homodimers or as heterodimers. However, our previous observations demonstrated that RXR was diffusely distributed in unstimulated HUVECs, with exposure to high glucose resulting in nuclear localization of RXRα (Chai et al., 2008). Here, using RXR siRNA, we confirmed that RXR mediated the suppressive effects on NADPH oxidase-NF-κB. These data suggested that the cytoplasmic activity of RXR might be associated with the inactivation NADPH oxidase induced by high glucose.

Human endothelial cells express all classic NADPH oxidase subunits, including membrane-bound Nox4, gp91^{phox}, and p22^{phox} subunits, as well as cytosolic p47^{phox} and p67^{phox} subunits (Gorlach et al., 2000). It is possible that cytoplasmic RXR plays a role in inhibiting NADPH oxidase complex activation through direct protein-protein interaction or other undefined mechanism, which require further investigation. Antagonism of NF-κB is an important mechanism for the control of genes involved in inflammation, cell proliferation and apoptosis by nuclear receptors (De Bosscher et al., 2006; Kassel and Herrlich, 2007). Although ROS acts as intracellular signal to mediate the repressive effect of RXR ligands on high glucose-induced NF-κB activation, the exact mechanism underlying RXR antagonism of NF-κB remains to be established, but may involve crosstalk between RXR and NF-κB.

In conclusion, we show here that RXR agonists inhibit high glucose-induced inflammation in endothelial cells in part through RXR-mediated inhibition of activation of NADPH oxidase-NF-κB pathway. RXR ligands may represent a class of promising agents for preventing atherosclerosis resulted from high glucose.

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