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Rosiglitazone attenuates NF-κB-dependent ICAM-1 and TNF-α production caused by homocysteine via inhibiting $ERK_{1/2}$/p38MAPK activation

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Abstract

Previous studies demonstrated an important interaction between nuclear factor-kappaB (NF-κB) activation and homocysteine (Hcy)-induced cytokines expression in endothelial cells and vascular smooth muscle cells. However, the underlying mechanism remains illusive. In this study, we investigated the effects of Hcy on NF-κB-mediated sICAM-1, TNF-α production and the possible involvement of $ERK_{1/2}$/p38MAPK pathway. The effects of rosiglitazone intervention were also examined. Our results show that Hcy increased the levels of sICAM-1 and TNF-α in cultured human umbilical vein endothelial cells (HUVECs) in a time- and concentration-dependent manner. This effect was significantly depressed by rosiglitazone and different inhibitors (PDTC, NF-κB inhibitor; PD98059, MEK inhibitor; SB203580, p38MAPK specific inhibitor; and staurosporine, PKC inhibitor). Next, we investigated the effect of Hcy on $ERK_{1/2}$/p38MAPK pathway and NF-κB activity in HUVECs. The results show that Hcy activated both $ERK_{1/2}$/p38MAPK pathway and NF-κB–DNA-binding activity. These effects were markedly inhibited by rosiglitazone as well as other inhibitors (SB203580, PD98059, and PDTC). Further, the pretreatment of staurosporine abrogated $ERK_{1/2}$/p38MAPK phosphorylation, suggesting that Hcy-induced $ERK_{1/2}$/p38MAPK activation is associated with PKC activity. Our results provide evidence that Hcy-induced NF-κB activation was mediated by activation of $ERK_{1/2}$/p38MAPK pathway involving PKC activity. Rosiglitazone reduces the NF-κB-mediated sICAM-1 and TNF-α production induced by Hcy via inhibition of $ERK_{1/2}$/p38MAPK pathway.

Keywords: Homocysteine; MAPKs; sICAM-1; TNF-α; Rosiglitazone

The development and progress of atherosclerosis is considered to be a form of chronic inflammation [1]. One of the important features of atherosclerosis is the activation of express cell surface-adhesion molecules and some inflammatory cytokines, such as the intercellular-adhesion molecule (ICAM-1) and TNF-α [2,3]. Hyperhomocysteinemia is an independent risk factor for atherosclerosis [4]. In vitro studies have demonstrated that Hcy enhances the production of several pro-inflammatory cytokines, but the mechanism is not fully understood. There is emerging evidence that activation of NF-κB plays an important role in the transcriptional activation of numerous pro-atherogenic and pro-inflammatory genes [5].

Several signaling pathways including protein kinase C (PKC), protein tyrosine kinase (PTK), or mitogen-activated protein kinase (MAPKs) may be involved in the

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mediation of adhesion molecules or cytokines production in response to various stimuli. MAPKs are a family of serine/threonine kinases that regulate the diversity of cellular activities. Three major classes have been described: ERKs, JNKs, and p38MAPK. One of the ubiquitous intracellular signaling mechanisms is the ERK pathway. Two major isoforms of ERK, ERK_1 and ERK_2, have been identified in mammalian systems [6]. Previous studies have demonstrated that $ERK_{1/2}$/p38MAPK pathway plays an important role in Hcy-induced cytokine gene expression [7]. However, the direct link between MAPKs and NF-κB activation in Hcy-induced cytokine production has not been established.

Rosiglitazone is a *peroxisome proliferator-activated* receptor gamma (PPARγ) agonist. Moreover, PPARγ induces a decrease in pro-inflammatory cytokine expression by antagonizing the activities of c-jun-NH2-terminal kinase (JNK), ERK and p38MAPK in vivo [8], interferes with the transcription factor activation such as nuclear factor NF-κB, signal transducers and transcription activators (STAT), activating protein 1 (AP-1), and the nuclear factor of activated T-cells (NFAT), all of which regulate cytokine gene expression. We speculate that rosiglitazone may potentially antagonize the Hcy-induced NF-κB activation and the subsequent expression of target genes.

Therefore, the present study was designated to study the possible involvement of $ERK_{1/2}$/p38MAPK pathway in Hcy-induced NF-κB activation and subsequent sICAM-1, TNF-α production in cultured HUVECs. Furthermore, the effects of rosiglitazone intervention were also observed.

Materials and methods

Reagents

Homocysteine, Dimethyl sulfoxide (DMSO), and PDTC were obtained from Sigma. Rosiglitazone was obtained from Glaxo Smith Kline Company. DMEM was obtained from Gibco. sICAM-1 and TNF-α ELISA kits were obtained from Jingmei Biotech Co., Ltd. Gel shift assay system for determination of NF-κB activity, anti-IκB-α, and β-actin were obtained from Promega, anti-p38MAPK antibody (Upstate Company), and anti-$ERK_{1/2}$ antibody (RD Systems). SB 203580, PD98059, and staurosporine were purchased from Calbiochem Co. (La Jolla, CA).

Cell culture

HUVECs were cultured according standard procedure as described elsewhere. Cells were seeded at a density of 3×10^5 per 100-mm dish in DMEM, supplemented with 20 mM Hepes and 20% FCS. The cultures were maintained at 37 °C with a gas mixture of 5% CO_2/95% air. All media were supplemented with 5 U/ml heparin, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Medium was changed every 2–3 days. Endothelial cells of the forth to sixth passages in the actively growing condition were used for experiments.

Determination of TNF-α and sICAM-1 concentration

TNF-α and sICAM-1 level in the endothelial cell-conditioned medium were measured by enzyme-linked immunosorbent assay (ELISA). The measurement of both TNF-α and sICAM-1 was performed step-by-step based on the protocol booklet of ELISA kit (Jingmei Biotech Co., Ltd.). Briefly, the conditioned medium was incubated with mouse IgG monoclonal antibodies against sICAM-1 or TNF-α. Then, after incubation of the medium with a second biotinylated antibody, a streptavidin-biotinylated horseradish peroxidase complex was added. Orthophenylenediamine served as substrate for the horseradish peroxidase complex. The optical density values were measured at 490 nm by a microplate reader (Biotek). The standard curve for TNF-α measured by this ELISA was linear from 16 to 250 pg/ml. The standard curve for sICAM-1 measured by this ELISA was linear from 30 to 2000 pg/ml.

Electrophoretic mobility shift assay

Endothelial cells were seeded into 25 cm^2 culture flasks at an optimal density of 10^5 cells/ml. Cells were washed with PBS for twice and incubated with 400 μl buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min on ice. Then 25 μl of 10% NP-40 solution was added. After vortexing, cell lysates were centrifuged by 1500 rpm for 2 min at 4 °C and nuclei were resuspended in 50 μl of buffer B (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerin 1 mM DTT, 1 mM PMSF) and vigorously vortexed at 4 °C for 15 min. Nuclear lysates were next centrifuged at 14,000 rpm for 5 min and the supernatant containing the nuclear proteins was carefully removed. Protein aliquots were either frozen at −70 °C or immediately used for electrophoretic mobility shift assay (EMSA) as described. The protein concentration was measured using bicinchoninic acid assay kit with bovine serum albumin as standard (Pierce, Rockford, IL).

Oligonucleotide labeling. Oligonucleotides were labeled using a double-stranded consensus sequence NF-κB (3′-TCA ACT CCC CTG AAA GGG TCC G-5′; 5′-AGT TGA GGG GAC TTT CCC AGG C-3′) and [γ-^{32}P]ATP (Yahui, Beijing, China). The reaction was catalyzed with T4 polynucleotide kinase and incubated in 10× kinase buffer at 37 °C for 45 min. The reaction was stopped by heating at 65 °C for 10 min. The labeled probe was purified through Sephadex G-25.

Nuclear extracts were analyzed by EMSA. Binding reactions containing equal amounts of nuclear protein extracts (15 μg) and 35 fmol of oligonucleotide were performed in binding buffer (Promega). Reaction volumes were held constant to 20 μl. After 10 min of incubation at room temperature, the mixture was run on a 4% non-denaturing polyacrylamide gel in 0.5× TBE buffer. After electrophoresis, the gels were dried and the DNA–protein complexes were detected by autoradiography. Specificity was determined by addition of an excess of unlabeled NF-κB oligonucleotide or unlabeled AP-2 oligonucleotide to the nuclear extracts before formation of DNA–protein complexes.

Western immunoblotting analysis of IκB-α and $ERK_{1/2}$/p38MAPK protein

The cellular levels of IκB-α and $ERK_{1/2}$/p38MAPK protein were determined by Western immunoblotting analysis. Briefly, cell proteins were separated by SDS/12.5%(w/v)–PAGE, followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. The membrane was probed with rabbit anti-IκB-α antibody, anti-p38MAPK antibody or anti-$ERK_{1/2}$ antibody. Bands corresponding to IκB-α and $ERK_{1/2}$/p38MAPK were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst2 version 1.1).

Statistic analysis

Results are expressed as means ± SEM. Data were analyzed by ANOVA followed by the unpaired Student's *t*-test for multiple comparisons. The significance level was chosen as $P < 0.05$.

Results

Hcy-induced sICAM-1 and TNF-α production in cultured endothelial cells and the effects of rosiglitazone or different inhibitors treatment

We first examined the time course and concentration dependence of Hcy-induced sICAM-1 and TNF-α production in cultured HUVECs. The cells were exposed to Hcy for different time periods (0, 6, 12, 24, and 48 h) and of different concentrations (10, 30 or 100 μM). Time course experiment revealed that Hcy time-dependently increased sICAM-1 and TNF-α production and both reached the peak at 24–48 h (Fig. 1A). Hcy also concentration-dependently increased sICAM-1 and TNF-α production from 10 to 100 μM (Fig. 1B). Next, we investigated the effects of rosiglitazone (10, 30, and 100 μM) or different inhibitors treatment on Hcy-induced sICAM-1 and TNF-α elevation. The results showed that rosiglitazone concentration-dependently reduced sICAM-1, TNF-α elevation induced by Hcy (100 μM). Further, pretreatment with PD98059 (50 μM), SB203580 (20 μM), PDTC (100 μM), and staurosporine (10 μM) all prevented Hcy-induced sICAM-1, TNF-α production, and that rosiglitazone (100 μM) itself had no effect on sICAM-1 and TNF-α production in cultured HUVECs (Fig. 1C). These data suggest that the activation of PKC, MAPK, and NF-κB is involved in the Hcy-induced secretion of sICAM-1 and TNF-α.

Effect of Hcy on the $ERK_{1/2}$/p38MAPK activation in cultured endothelial cells

HUVECs were exposed to Hcy for different time periods (0, 10, 20, and 30 min) or of different concentrations (10, 30 or 100 μM). The results revealed that $ERK_{1/2}$/p38MAPK was time- and concentration-dependently activated by Hcy (Fig. 2A and B). Maximal phosphorylation of $ERK_{1/2}$ and p38MAPK was obtained at 30 min in HUVECs treated with Hcy (100 μM). Pretreatment with rosiglitazone (10, 30, and 100 μM) inhibited $ERK_{1/2}$/p38MAPK activation induced by Hcy (100 μM) in a concentration-dependent manner. To verify the signing pathway, specific inhibitors were used: PD98059 (inhibits the upstream activator of $ERK_{1/2}$); SB203580 (inhibits the kinase activity of p38MAPK); staurosporine (inhibits PKC activity); and *PDTC* (inhibits the activation of NF-κB). As shown in the Fig. 2C, Hcy-induced phosphorylation of $ERK_{1/2}$/p38MAPK was markedly reduced by staurosporine, PD98509, and SB203580, however, PDTC had no effect on these events.

Hcy activates NF-κB–DNA-binding activity

To investigate whether Hcy stimulated NF-κB–DNA-binding activity, cells were incubated with Hcy (100 μM) for various time periods (0, 0.5, 1, 2, and 4 h) and of differ-

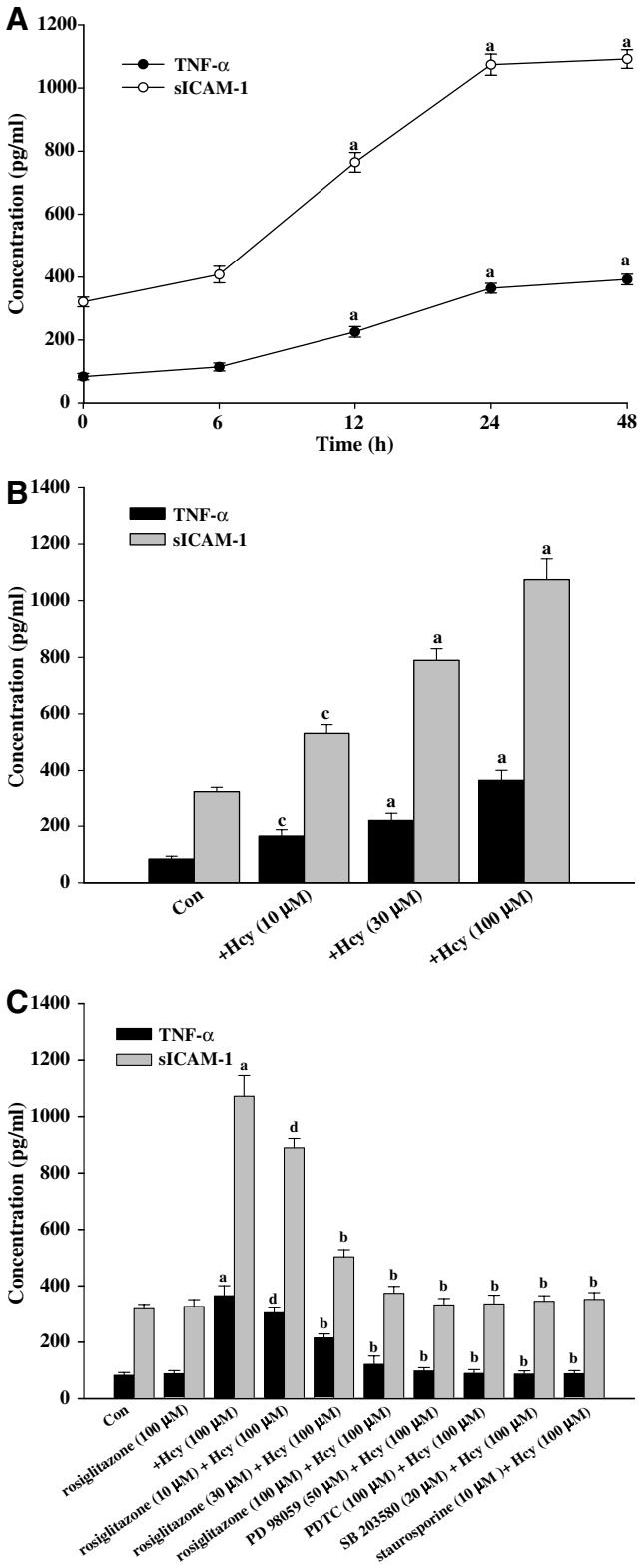

Fig. 1. (A) Time course of Hcy-induced sICAM-1 and TNF-α production in cultured HUVECs. The cells were exposed to Hcy (100 μM) for 0, 6, 12, and 24 h. (B) Concentration dependence of Hcy-induced sICAM-1 and TNF-α production in cultured HUVECs. The cells were exposed to Hcy of different concentrations (10, 30, and 100 μM) for 24 h. (C) The effect of rosiglitazone (10, 30, and 100 μM) and different inhibitors on Hcy-induced sICAM-1 and TNF-α production in cultured HUVECs. Values are means ± SEM from three different experiments. $^{a,c}P < 0.01$ or 0.05 vs. control, $^{b,d}P < 0.01$ or 0.05 vs. Hcy treatment.

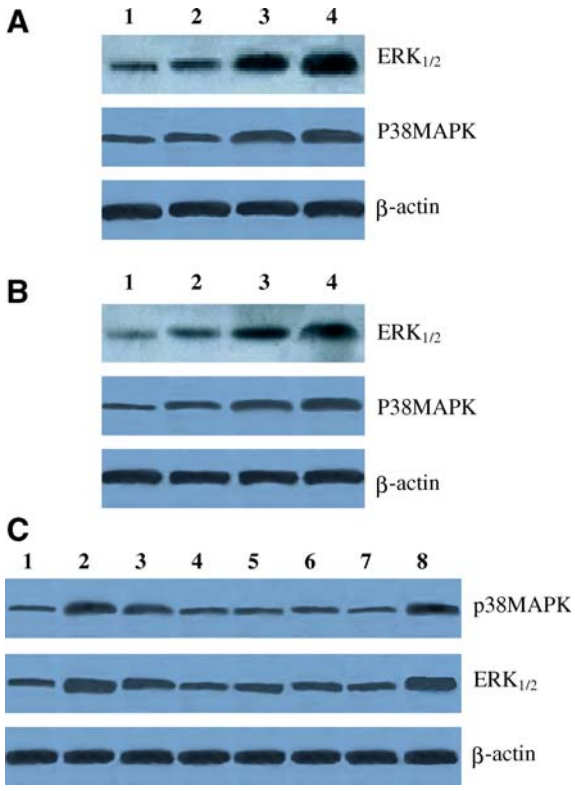

Fig. 2. (A) Time course of Hcy-induced phosphorylation of $ERK_{1/2}$/p38MAPK in cultured HUVECs. (1) 0 min, (2) 10 min, (3) 20 min, (4) 30 min. (B) Concentration dependence of Hcy-induced phosphorylation of $ERK_{1/2}$/p38MAPK in cultured HUVECs. (1) control, (2) Hcy 10 μM, (3) Hcy 30 μM, (4) Hcy 100 μM. (C) The effect of rosiglitazone (10, 30, and 100 μM) and different inhibitors on Hcy-induced phosphorylation of $ERK_{1/2}$/p38MAPK in cultured HUVECs. (1) control, (2) Hcy (100 μM), (3) rosiglitazone (10 μM) + Hcy (100 μM), (4) rosiglitazone (30 μM) + Hcy (100 μM), (5) rosiglitazone (100 μM) + Hcy (100 μM), (6) staurosporine (10 μM) + Hcy (100 μM), (7) SB 203580 (20 μM) or PD98059 (50 μM) + Hcy (100 μM), (8) PDTC (100 μM) + Hcy (100 μM).

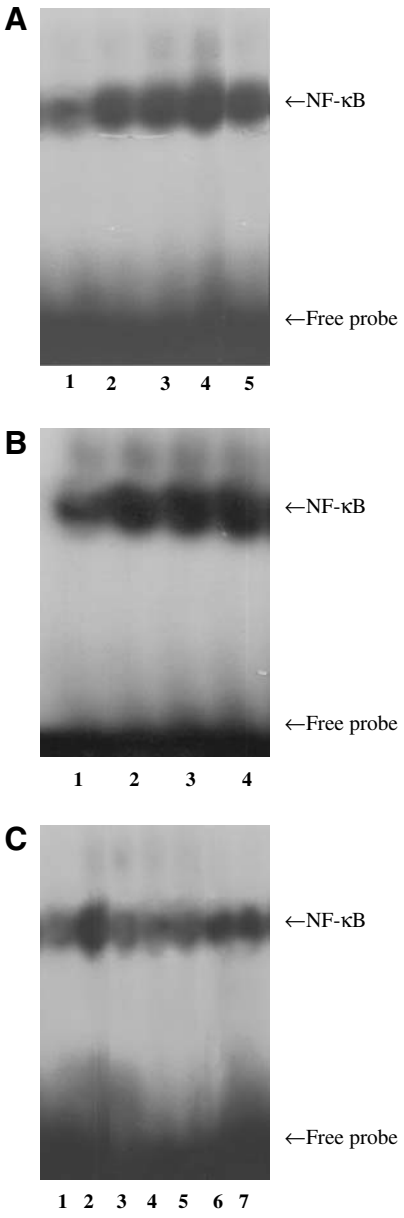

Fig. 3. (A) Time course of Hcy-induced NF-κB/DNA-binding activity in cultured HUVECs. (1) 0 h, (2) 0.5 h, (3) 1 h, (4) 2 h, (5) 4 h. (B) Concentration dependence of Hcy-induced NF-κB/DNA-binding activity in cultured HUVECs. (1) control, (2) Hcy (10 μM), (3) Hcy (30 μM), (4) Hcy (100 μM). (C) The effect of rosiglitazone and different inhibitors on Hcy-induced NF-κB/DNA-binding activity in cultured HUVECs. (1) control, (2) Hcy (100 μM), (3) rosiglitazone (100 μM) + Hcy (100 μM), (4) PDTC (100 μM) + Hcy (100 μM), (5) staurosporine (10 μM) + Hcy (100 μM), (6) PD98059 (50 μM) + Hcy (100 μM), (7) SB203580 (20 μM) + Hcy (100 μM).

ent concentrations (10, 30 or 100 μM). As shown in Fig. 3A, Hcy significantly stimulated the NF-κB–DNA-binding activity in endothelial cells at 0.5 h and was maintained up to 2 h. Increasing of the NF-κB–DNA-binding activity was observed in Hcy-treated (10, 30, and 100 μM) cells (Fig. 3B). As shown in the Fig. 3C, rosiglitazone (100 μM) markedly inhibited NF-κB–DNA-binding activity induced by Hcy (100 μmol/L), pretreatment with the inhibitors of PKC (staurosporine, 10 μM), MEK (PD98059, 50 μM), p38MAPK (SB203580, 20 μM), or NF-κB (PDTC, 100 μM) also inhibited NF-κB–DNA-binding activity.

Effects of Hcy on IκB-α level in endothelial cells

The activation of NF-κB might be caused by enhanced phosphorylation of the inhibitor protein IκB-α. Therefore the effect of Hcy on the phosphorylation status of IκB-α was investigated. As shown in Fig. 4A, Hcy treatment caused a significant decrease in the level of IκB-α protein in HUVECs at 0.5 h and the maximum inhibitory effect was obtained from cells treated with Hcy at 2 h. Hcy (10, 30, and 100 μM) concentration-dependently increased the IκB-α phosphorylation in endothelial cells (Fig. 4B). As shown in the Fig. 4C, rosiglitazone (100 μM) significantly inhibited the decrease of IκB-α level induced by Hcy (100 μM), the same effects were also observed when the cells pretreated with PDTC, staurosporine, PD98509, and SB203580.

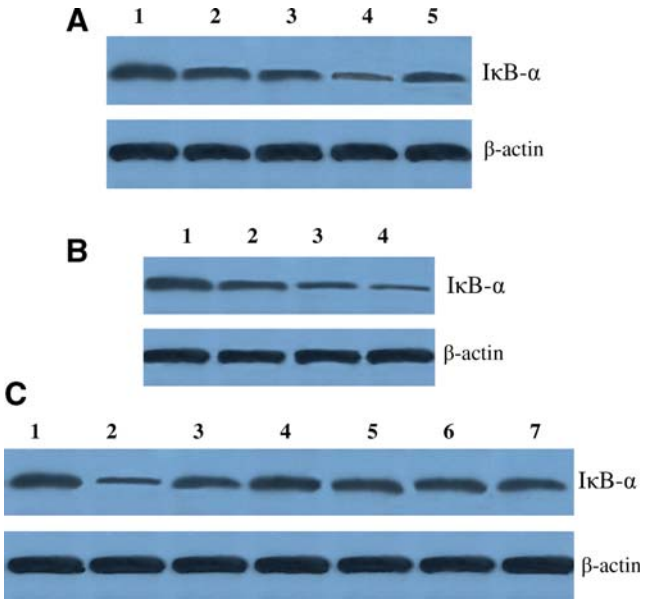

Fig. 4. (A) Time course of Hcy-induced IκB-α phosphorylation and degradation in cultured HUVECs. (1) 0 h, (2) 0.5 h, (3) 1 h, (4) 2 h, (5) 4 h. (B) Concentration dependence of Hcy-induced IκB-α phosphorylation and degradation in cultured HUVECs. (1) control, (2) Hcy (10 μM), (3) Hcy (30 μM), (4) Hcy (100 μM). (C) The effect of rosiglitazone and different inhibitors on Hcy-induced IκB-α phosphorylation and degradation in cultured HUVECs. (1) control, (2) Hcy (100 μM), (3) rosiglitazone (100 μM) + Hcy (100 μM), (4) PDTC (100 μM) + Hcy (100 μM), (5) staurosporine (10 μM) + Hcy (100 μM), (6) PD98059 (50 μM) + Hcy (100 μM), (7) SB203580 (20 μM) + Hcy (100 μM).

Discussion

Numerous epidemiological studies have demonstrated that elevated plasma level of Hcy is an independent risk factor for the development of cardiovascular diseases. In vitro studies have shown that Hcy can directly impair endothelial cell, increase proliferation of smooth muscle cells and induce pro-inflammatory response [9,10]. However, the mechanisms underlying Hcy-induced vascular inflammation are not well understood.

The results obtained from the present study have demonstrated that Hcy stimulates sICAM-1 and TNF-α expression in HUVECs, and such stimulatory effect is mediated by activation of NF-κB; PKC and $ERK_{1/2}$/p38MAPK pathway may participate in the upstream signal transduction of NF-κB activation; Rosiglitazone attenuated the increases of sICAM-1 and TNF-α induced by Hcy via inhibiting the $ERK_{1/2}$/p38MAPK pathway and activation of NF-κB.

The PKC family is thought to play a role in monocyte–endothelial interaction and several studies have found that inhibition of PKC activation is associated with the reduction of monocyte adhesion to endothelial cells [11,12]. The results from present study showed that the activation of PKC is necessary for both sICAM-1 and TNF-α production induced by Hcy, because staurosporine, a PKC inhibitor, significantly reduced sICAM-1 and TNF-α production in cultured HUVECs. Many previous studies have demonstrated that Hcy stimulates MAPKs activation, and causes activation of JNK in vascular endothelial cells [13,14]. MAPKs represent a family of eukaryotic protein kinases involved in various cellular processes. Three parallel cascades (p38MAPK, ERK, and JNK) are now commonly described. As an upstream signaling molecule, the activation of PKC may mediate the activation of MAPKs, such as MEK-$ERK_{1/2}$, p38MAPK, and JNK [7,15]. Thus, we investigated specific downstream signaling molecules that could be potentially important as the targets of PKC. Our results have demonstrated that MAPKs activation is induced by Hcy with the maximal level at 30 min. In order to determine whether $ERK_{1/2}$/p38MAPK is activated via the Raf-MEK-$ERK_{1/2}$ and PKC pathway, we used the specific inhibitors (PD98509, SB203580, and staurosporine) which markedly reduced MAPKs activation, suggesting that Hcy-induced MAPKs activation is MEK- or PKC-dependent. We also found that PD98509, SB203580, and staurosporine significantly decreased sICAM-1 and TNF-α production. These data are consistent with other reports that MAPKs are required for adhesion molecules production in several other cell systems in response to various stimuli [16,17].

NF-κB has been implicated to play a vital role in the initiation and development of atherosclerosis. NF-κB is normally present in the cytoplasm in an inactive form associated with an inhibitory protein named IκB and that IκB-α is the best-characterized form of IκB. Upon stimulation with various NF-κB stimuli, IκB-α is rapidly phosphorylated, leading to the ubiquitination and subsequent degradation of IκB-α, as well as translocation of NF-κB to the nucleus [5]. Many studies suggest that NF-κB plays an important role in upregulating the expression of many inflammatory factors. The activation of NF-κB is necessary for Hcy-induced cytokines expression such as MCP-1, IL-1, and IL-6 [18–20]. The results from present study indicate that NF-κB is activated in Hcy-treated endothelial cells. First, the results from EMSA demonstrated that activation of NF-κB by Hcy treatment (maximal level at 2 h) preceded the elevation of sICAM-1 and TNF-α (maximal level at 24 h). Second, the results showed that PDTC completely abolished sICAM-1 and TNF-α expression induced by Hcy. The pathways of NF-κB activation have been intensely elucidated by many investigators. Most studies have demonstrated that upon stimulation, after IκB-α is rapidly phosphorylated and then degraded. Released NF-κB can then translocate to the nucleus and regulate the expression of target genes. In the present study, the levels of IκB-α protein in endothelial cells treated with Hcy decreased significantly after 30 min and reached the lowest at 2 h. Therefore, the NF-κB activation might serve as a signal after $ERK_{1/2}$/p38MAPK activation during the inducement of ICAM-1 and TNF-α expression by Hcy.

Recently, PPARγ has been described as a regulator of cellular proliferation, apoptosis, and anti-inflammatory response [21,22]. PPARγ activators inhibited expression

of VCAM-1 and ICAM-1 which reduce monocyte/macrophage homing to atherosclerotic plaques in activated endothelial cells [23]. Jiang et al. [24] reported that incubation of human monocytes with the natural PPARγ ligand, or with synthetic agonists, inhibited the production of pro-inflammatory cytokines. Other studies showed that PPARγ inhibited the transcriptional activity of genes by interfering with transcription factors such as NF-κB [25]. Hernandez1 et al. [26] reported that rosiglitazone inhibited TNF-α-induced p38MAPK and $ERK_{1/2}$ activation. Souza [27] found that rosiglitazone blocked TNF-α-induced ERK but not JNK activation in 3T3-L1 adipocytes, resulting in reduced lipolysis. Subramanyam et al. [28] had shown that rosiglitazone reduced serum Hcy levels, smooth muscle proliferation and intimal hyperplasia in rats by fed a high methionine. In the present study, cultured endothelial cell was pretreated with rosiglitazone then exposed to Hcy, the results show that rosiglitazone depressed the MAPKs and NF-κB activation, decreased the sICAM-1 and TNF-α production induced by Hcy. It furnishes, to our knowledge, the first evidence that rosiglitazone has direct antagonizing effect on Hcy-induced inflammatory responses apart from it reducing the elevation of serum Hcy level.

In conclusion, the present study indicates that rosiglitazone inhibited the activation of NF-κB and decreased the levels of sICAM-1 and TNF-α induced by Hcy via inhibiting $ERK_{1/2}$/p38MAPK pathway. These findings may provide us with one of the important mechanisms of Hcy caused atherosclerosis and provide a novel approach to prevent the pro-atherogenic effect of Hcy.

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