Basic Study

High levels of homocysteine downregulate apolipoprotein E expression *via* nuclear factor kappa B

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Abstract

AIM: To investigate the effect of high homocysteine (Hcy) levels on apolipoprotein E (apoE) expression and the signaling pathways involved in this gene regulation.

METHODS: Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot were used to assess apoE expression in cells treated with various concentrations (50-500 μmol/L) of Hcy. Calcium phosphate-transient transfections were performed in HEK-293 and RAW 264.7 cells to evaluate the effect of Hcy on apoE regulatory elements [promoter and distal multienhancer 2 (ME2)]. To this aim, plasmids containing the proximal apoE promoter [(-500/+73)apoE construct] alone or in the presence of ME2 [ME2/(-500/+73)apoE construct] to drive the expression of the reporter luciferase gene were used. Co-transfection experiments were carried out to investigate the downstream effectors of Hcy-mediated regulation of apoE promoter by using specific inhibitors or a dominant negative form of IKβ. In other co-transfections, the luciferase reporter was under the control of synthetic promoters containing multiple specific binding sites for nuclear factor kappa B (NF-κB), activator protein-1 (AP-1) or nuclear factor of activated T cells (NFAT). Chromatin immunoprecipitation (ChIP)

assay was accomplished to detect the binding of NF-κB p65 subunit to the apoE promoter in HEK-293 treated with 500 μmol/L Hcy. As control, cells were incubated with similar concentration of cysteine. NF-κB p65 proteins bound to DNA were immunoprecipitated with anti-p65 antibodies and DNA was identified by PCR using primers amplifying the region -100/+4 of the apoE gene.

RESULTS: RT-PCR revealed that high levels of Hcy (250-750 μmol/L) induced a 2-3 fold decrease in apoE mRNA levels in HEK-293 cells, while apoE gene expression was not significantly affected by treatment with lower concentrations of Hcy (100 μmol/L). Immunoblotting data provided additional evidence for the negative role of Hcy in apoE expression. Hcy decreased apoE promoter activity, in the presence or absence of ME2, in a dose dependent manner, in both RAW 264.7 and HEK-293 cells, as revealed by transient transfection experiments. The downstream effectors of the signaling pathways of Hcy were also investigated. The inhibitory effect of Hcy on the apoE promoter activity was counteracted by MAPK/ERK kinase 1/2 (MEK1/2) inhibitor U0126, suggesting that MEK1/2 is involved in the downregulation of apoE promoter activity by Hcy. Our data demonstrated that Hcy-induced inhibition of apoE took place through activation of NF-κB. Moreover, we demonstrated that Hcy activated a synthetic promoter containing three NF-κB binding sites, but did not affect promoters containing AP-1 or NFAT binding sites. ChIP experiments revealed that NF-κB p65 subunit is recruited to the apoE promoter following Hcy treatment of cells.

CONCLUSION: Hcy-induced stress negatively modulates apoE expression *via* MEK1/2 and NF-κB activation. The decreased apoE expression in peripheral tissues may aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions.

Key words: Apolipoprotein E; Homocysteine; Nuclear factor kappa B; Gene regulation; MAPK/ERK kinase

Core tip: This original manuscript investigates the effect of high homocysteine (Hcy) levels on apoE expression, and the signaling pathways involved in this gene regulation. Our novel findings show that high doses of Hcy decrease apolipoprotein E (apoE) expression. We revealed that this regulation involves nuclear factor kappa B activation, *via* MAPK/ERK kinase. The Hcy-mediated decrease of apoE expression in peripheral tissues may aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions. Thus, the current manuscript may be of interest for scientists working in the field of cardiovascular disease and related inflammatory disorders.

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INTRODUCTION

Cardiovascular disease, the main cause of death in Western world, is contributed by both genetic and environmental risk factors. Among these, hyperhomocysteinemia (HHcy) has received extensive attention since it has been hypothesized to be an independent risk factor for atherosclerosis[1]. However, the first report of the association between genetically induced high plasma level of homocysteine (Hcy) and premature arterial lesions belongs to McCully *et al*[2]. Hcy is a key intermediate in the metabolic pathway of the sulfur-containing amino acids leading from exogenous essential methionine to cysteine. Whether a cause or a marker for vascular pathology, HHcy remains strongly associated with alterations such as endothelial dysfunction, smooth muscle cells proliferation, increased inflammation, plaque instability[3]. Many of these alterations act in a concerted fashion toward an atherogenic phenotype. Thus, HHcy induces upregulation of PDGF production by endothelial cells, which in turn promotes proliferation and migration of co-cultured vascular smooth muscle cells[4]. Hcy accelerates senescence and reduces the endothelial progenitor cells proliferation through telomerase inactivation and Akt dephosphorylation[5].

Overall, the effects of elevated Hcy are multifactorial, affecting both the blood vessel structure and the coagulation cascade. Not only the arterial vasculature is challenged by increased Hcy, but also the brain, the bone, the kidney, and blood cells. Neurodegenerative diseases such as Alzheimer's[6] and Parkinson's[7] are associated with HHcy. Elderly individuals with high plasma Hcy are susceptible to hip fractures, exhibiting reduced bone mineral density, alterations of collagen stability due to diminished cross-linking and impaired bone blood flow[8].

Hcy may exert its action through several mechanisms, such as production of reactive oxygen species[9], which may also lead to decreased nitric oxide availability[10], endoplasmic reticulum stress[11], gene regulation[12-14], epigenetic changes such as altered DNA methylation patterns[15], altered protein modification (*e.g.,* N-homocysteinylation of fibrinogen)[16].

Apolipoprotein E (apoE) is an anti-atherogenic 35 kDa glycoprotein associated with all classes of lipoproteins, and it is synthesized by the liver and different peripheral sources, such as macrophages, adipocytes, astrocytes, kidney cells[17]. ApoE plays an important role in lipid metabolism, atherogenesis, neurodegenerative disorders, inflammation, *etc*[17-19]. ApoE gene regulatory mechanisms discovered so far have been recently reviewed[20]. Nevertheless, no reports exist so far regarding the gene regulation of apoE under HHcy stress. It is

only known from a recent report that at posttranslational level, HHcy exerts an inhibitory effect on *in vivo* and *in vitro* dimerization of apoE directly *via* the thiol group[21]. Herein, we investigated how increased Hcy levels affected apoE expression and the mechanisms involved in this modulation. The results obtained in the present study demonstrated that Hcy inhibits apoE expression and that this negative effect is mediated *via* nuclear factor kappa B (NF-κB) activation.

MATERIALS AND METHODS

Chemicals
Homocysteine was from Sigma-Aldrich (St. Louis, MO, United States). DMEM and fetal calf serum were obtained from EuroClone (Milano, Italy). M-MLV reverse transcriptase, oligo(dT), GoTaq DNA polymerase, Luciferase Assay System, U0126, SP600125 and N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhibitors were obtained from Promega Corp. (Madison, WI). Primers and TRIzol reagent were from Invitrogen Life Technologies (Carlsbad, CA). Anti-apoE and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States).

Plasmids
The construct (-500/+73)apoE-luc containing the apoE proximal promoter as well as the construct ME2/(-500/+73)apoE in which ME2 was cloned in front of the proximal apoE promoter in pGL3 basic vector were previously described[22]. The expression vectors for IKKβ, IKβ dominant negative (DN) and fusion hetero-dimer p65/p50, as well as the (NF-κB)$_3$-luc construct containing three NF-κB binding sites, and the (AP-1)$_7$-luc construct containing seven AP-1 binding sites, were kindly supplied by Prof. D. Kardassis (University of Crete Medical School, Greece).

Cell culture
RAW 264.7 and HEK-293 cells (both from ATCC) were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics.

Reverse transcription-polymerase chain reaction
Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. After reverse transcription, polymerase chain reaction (PCR) was performed using the following specific primers: Human apoE forward, 5´-CCAGCGGAGGTGAAGGAC; human apoE reverse, 5´-CGCTTCTGCAG GTCATCG; human β-actin forward 5´-CAACCGCGAGAAGATGACCC; human β-actin reverse, 5´-GGAAGGAAGGCTGGAAGAGTGC. The fragments obtained by Reverse Transcription-Polymerase chain reaction (RT-PCR) were of 584 bp for human apoE, and 458 bp for β-actin. The mRNA levels of β-actin were used to normalize the *apoE* gene expression.

Transient transfections
RAW 264.7 and HEK-293 cells were transiently transfected by Ca$_3$(PO$_4$)$_2$ co-precipitation method[23] with the above mentioned plasmids, containing promoters driving the luciferase reporter. Luciferase assay was performed using the Luciferase Assay Kit from Promega. Promoter activity was normalized to the activity of the co-transfected β-galactosidase, determined using o-nitrophenyl β-D-galactopyranoside as a substrate.

Immunoblotting
Cells were incubated with various concentrations of homocysteine for 24 h, and then cell lysates were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred onto nitrocellulose. The blots were probed with anti-apoE or anti-β-actin antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected using Super Signal West Pico chemiluminescent substrate (Pierce).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) experiments were performed using chromatin prepared from HEK-293 cells treated with 500 μmol/L homocysteine or cysteine for 24 h. Briefly, the cells were fixed with p-formaldehyde and the chromatin was sheared by sonication; p65 proteins bound to DNA were immuno-precipitated with anti-p65 antibodies and DNA was identified by PCR using primers amplifying the region -100/+4 of the apoE gene with the following primers: Forward 5´-ACCTCGTGACTGGGGGCTG, and reverse 5´-GCTGGGGCTGAGTAGGAC. The PCR products were analyzed by agarose gel electrophoresis.

Statistical analysis
Results are means ± SD of triplicate experiments. One-way analysis of variance was performed using OriginPro 7.5. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

ApoE expression is downregulated by homocysteine in HEK-293 cells
We investigated the modulation of apoE expression at mRNA and protein levels in HEK-293 cells, in response to various concentrations of homocysteine. The apoE gene expression in HEK-293 cells exposed to different concentrations of homocysteine (50-750 μmol/L) for 24 h was determined by RT-PCR using primers specific for apoE, and the results were normalized to actin expression. Our experiments showed that homocysteine concentrations of 250-750 μmol/L induced a 2-3 fold decrease of apoE mRNA levels in HEK-293 cells (Figure 1A), while lower concentrations of homocysteine (100 μmol/L) had no significant effect on apoE gene expression ($P > 0.05$) as compared to the control.
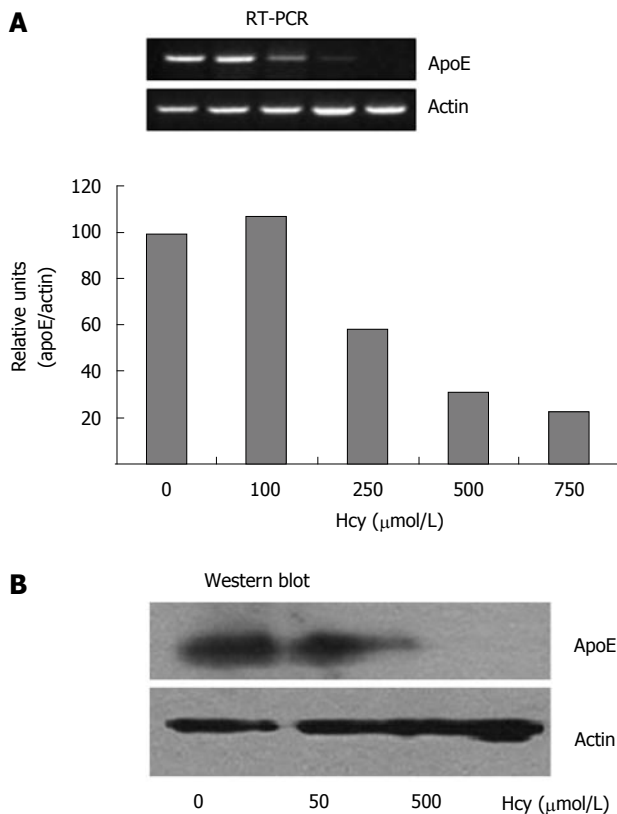
A

Figure 1 High homocysteine doses downregulate apoE expression in HEK-293 cells. ApoE expression in HEK-293 cells exposed for 24 h to increasing concentrations of homocysteine (50-750 μmol/L) was assessed by RT-PCR and immunoblotting. A: High homocysteine concentrations (250-750 μmol/L) significantly decreased apoE mRNA levels in HEK-293 cells; in contrast, lower doses of homocysteine (100 μmol/L), did not affect the expression level as compared to the control cells. The expression of β-actin was used for normalization; B: Treatment of HEK-293 cells with 500 μmol/L homocysteine, but not with 50 μmol/L homocysteine, dramatically reduced apoE protein expression. RT-PCR: Reverse transcriptase polymerase chain reaction; ApoE: Apolipoprotein E.

Next, we investigated the influence of Hcy treatment on apoE protein expression. HEK-293 cells were exposed to homocysteine (50-500 μmol/L) for 24 h and the endogenous levels of apoE protein were determined by immunoblotting using anti-apoE antibodies. Actin level was used as a control for the total protein load. Our data showed that apoE protein expression was not affected by 50 μmol/L Hcy (as compared to control cells), while it was dramatically reduced in cells exposed to 500 μmol/L Hcy (Figure 1B).

ApoE gene regulatory elements are affected by high homocysteine levels

The ability of homocysteine to modulate the transcriptional activity of apoE promoter was investigated by transient transfection experiments in HEK-293 and RAW 264.7 cells. For this purpose, cells were transiently transfected with plasmids containing apoE proximal promoter [(-500/+73)apoE construct] or plasmids in which multienhancer ME2 was cloned in front of the proximal apoE promoter [ME2/(-500/+73)apoE

construct]. After 18 h, the transfected cells were treated with various concentrations of homocysteine (50-500 μmol/L) and the next day, the reporter gene activity was determined by luciferase assay according to the manufacturer's instructions (Promega). As presented in Figure 2, the treatment of cells with 500 μmol/L Hcy significantly decreased ($P < 0.0025$) the apoE promoter activity, in the presence (Figure 2B and D) or in the absence of ME2 (Figure 2A and C), in a dose dependent manner, in both RAW 264.7 (Figure 2A and B) and HEK-293 cells (Figure 2C and D). Treatment of RAW 264.7 or HEK-293 cells with low concentrations of Hcy (50 μmol/L) did not significantly affect ($P > 0.05$) the activity of apoE promoter either in the absence (Figure 2A and C) or in the presence of ME2 (Figure 2B and D).

Taken together, these experiments clearly indicated that high concentrations of homocysteine exerted a negative effect on apoE expression. Therefore, we next sought to investigate the mechanism underlying this inhibitory effect and to determine the signaling pathways possibly involved.

Molecular mechanism of action of high Hcy levels on apoE gene regulation

To test whether MAPK signaling pathway is involved in the Hcy-mediated downregulation of apoE promoter activity, HEK-293 cells were transiently transfected with the construct (-500/+73)apoE-luc in the presence or in the absence of MEK1 expression vectors or MEK inhibitor U0126 (10 μmol/L). The results of these experiments showed that MEK1 overexpression, as well as the treatment of cells with Hcy, significantly decreased ($P < 0.05$) the activity of apoE proximal promoter, as compared to the control (Figure 3, lane Hcy and lane MEK1). Notably, MEK inhibitor U0126 counteracted the inhibitory effect of Hcy on the apoE promoter activity (Figure 3, lane Hcy + U0126). Therefore, our data suggest that MEK1/2 is involved in Hcy-mediated downregulation of apoE promoter activity.

To determine whether Hcy modulates the apoE promoter activity through NF-κB signaling, cells transiently transfected with (-500/+73)apoE–luc construct were treated with Hcy simultaneously with NF-κB pathway inhibition. For this, either TPCK or overexpression of a DN form of IKβ was used. As shown in Figure 4, TPCK abrogated the inhibitory effect of Hcy on apoE promoter activity, similarly with the overexpression of IKβ DN. Moreover, the activity of apoE proximal promoter was significantly reduced ($P < 0.05$) by IKKβ overexpression, to a similar extent as Hcy treatment. Together, these data demonstrate the involvement of the NF-κB signaling pathway in the regulation of apoE promoter activity by homocysteine.

To evaluate the ability of homocysteine to activate NF-κB, HEK-293 cells were transiently transfected with (NF-κB)₃-luc construct containing three NF-κB binding sites. The transfected cells were treated with various concentrations of homocysteine (50-500 μmol/L) for

A

RAW 264.7

-500apoE — luc

C

HEK-293

-500apoE — luc

B

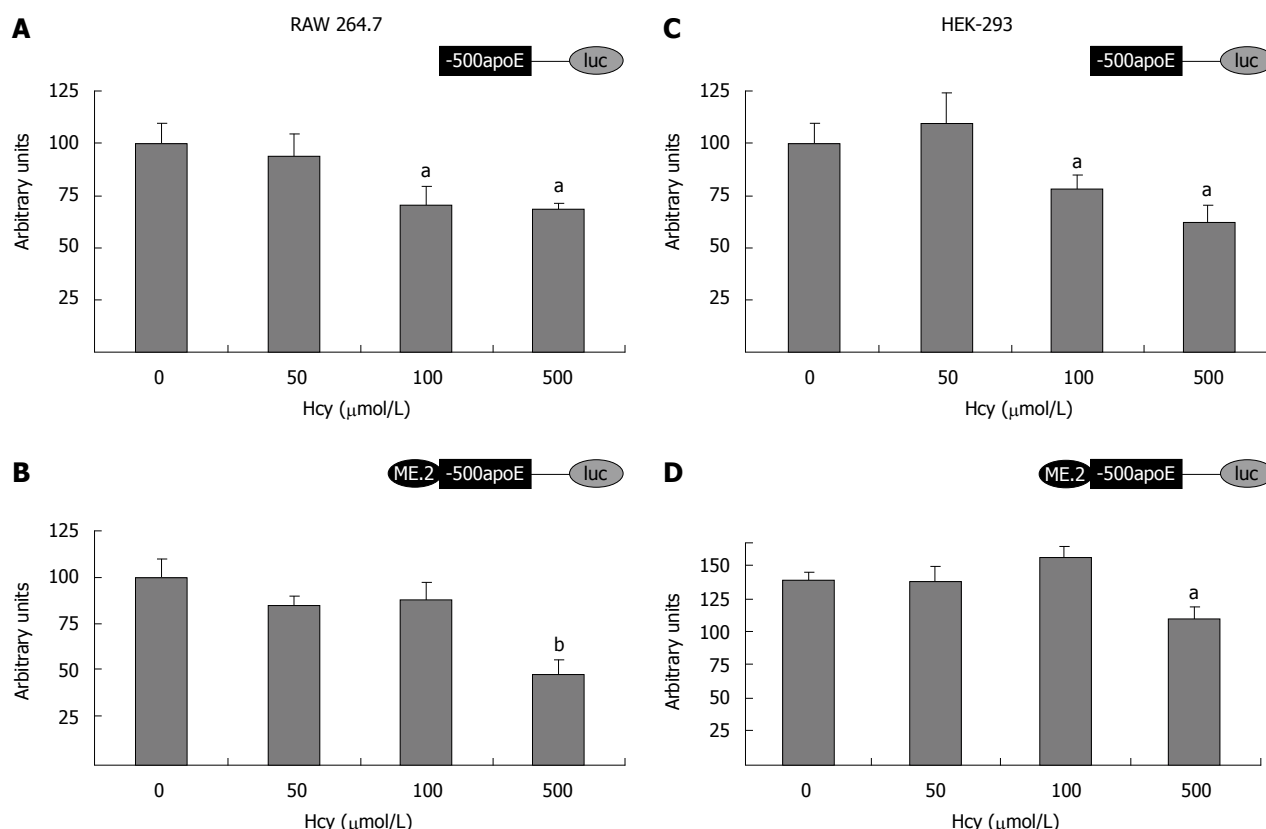ME.2 -500apoE — luc

D

ME.2 -500apoE — luc

Figure 2 Homocysteine inhibits the activity of apolipoprotein E proximal promoter in either absence or presence of ME2 in RAW 264.7 macrophages and HEK-293 cells. Transient transfection experiments were performed using (-500/+73) apoE proximal promoter (A, C) or ME2/(-500/+73) multienhancer/apoE promoter constructs (B, D) driving the luciferase reporter and the transfected cells were incubated with 50-500 μmol/L homocysteine for 24 h. Then, luciferase activity was assessed and normalized to the activity of the co-transfected β-galactosidase. Treatment of RAW 264.7 (A, B) and HEK-293 cells (C, D) with 500 μmol/L Hcy significantly decreased ([a]$P < 0.05$ or [b]$P < 0.01$) apoE promoter activity, in the presence (B, D) or in the absence of ME2 (A, C). Low concentrations of Hcy (50 μmol/L) did not considerably decrease ($P > 0.05$) the activity of apoE promoter either in the absence (A, C) or in the presence of ME2 (B, D). MEK1/2: MAPK/ERK kinase; Hcy: Homocysteine; ApoE: Apolipoprotein E.
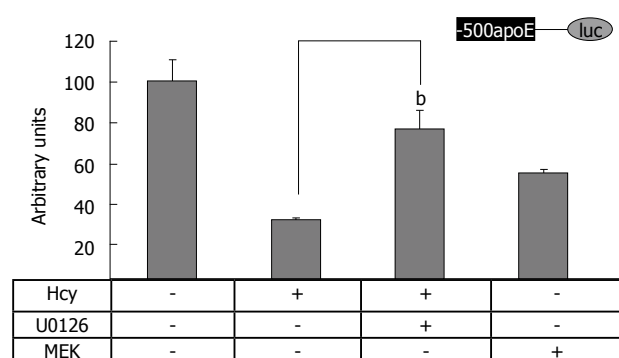
-500apoE — luc

Hcy	-	+	+	-
U0126	-	-	+	-
MEK	-	-	-	+

Figure 3 MEK1/2 is a downstream effector of Hcy action on apolipoprotein E promoter in HEK-293 cells. Transient transfections in HEK-293 cells were performed using plasmids containing apoE proximal promoter (-500/+73 region) in the presence or in the absence of homocysteine alone or in combination with U0126 (a MEK inhibitor), or MEK1 expression vectors. Hcy treatment as well as MEK1 overexpression significantly decreased ($P < 0.05$) the activity of (-500/+73) apoE-luc construct, as compared to the control. The decreased activity of the reporter luciferase gene driven by the apoE proximal promoter (-500/+73) in the presence of 500 μmol/L homocysteine was partially restored by 10 μmol/L U0126 ([b]$P < 0.01$). MEK1/2: MAPK/ERK kinase; Hcy: Homocysteine; ApoE: Apolipoprotein E.

24 h and then, the reporter gene activity was determined. Our data showed that cell treatment with high

concentrations of homocysteine (250 and 500 μmol/L) significantly increased ($P < 0.05$) the activity of the promoter containing the three NF-κB binding sites, while low concentrations of homocysteine (50 μmol/L) did not considerably affect ($P > 0.05$) its activity (Figure 5A). As a positive control for NF-κB activity, a synthetic fusion p65/p50 heterodimer was co-transfected. As expected, the activity of the (NF-κB)₃-luc construct was greatly enhanced by p65/p50 overexpression (Figure 5B).

We subsequently tested whether homocysteine is able to modulate the activity of AP-1 complex or NFAT transcription factor. To this aim, HEK-293 cells were transiently transfected with (AP-1)₇-luc construct (containing seven AP-1 binding sites) or (NFAT)₃-luc construct (having three NFAT binding sites), and exposed to different Hcy concentrations (50-500 μmol/L). None of the Hcy concentrations tested affected the activity of AP-1 or NFAT constructs ($P > 0.05$).

Taken together, these results demonstrate that homocysteine downregulates the apoE promoter *via* NF-κB activation; however, AP-1 or NFAT transcription factors do not mediate Hcy effect on apoE transcription.

Considering our findings that revealed the involvement of NF-κB signaling pathway in Hcy-mediated
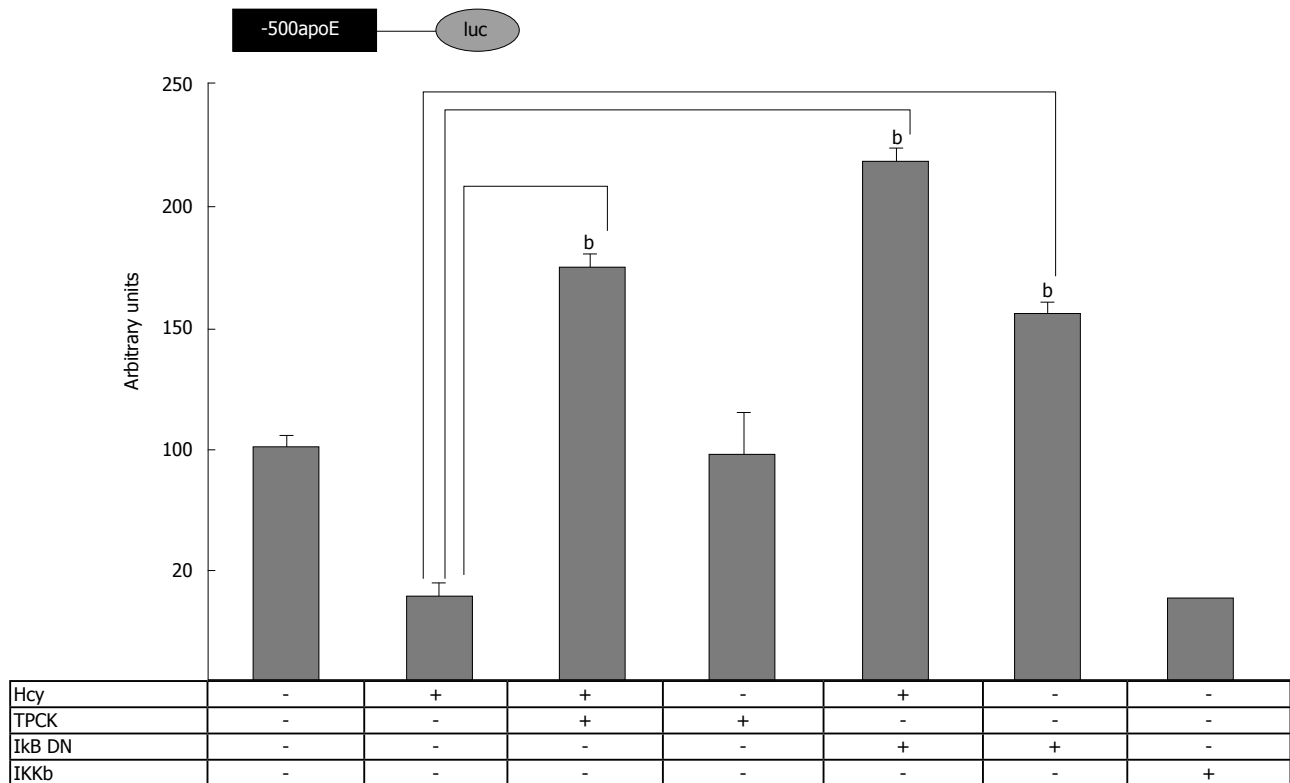
Figure 4 Involvement of nuclear factor kappa B signaling pathway in homocysteine-mediated inhibition of apolipoprotein E promoter activity. HEK-293 cells were transiently transfected with plasmids containing apoE proximal promoter in the presence of homocysteine, TPCK or expression vectors for IKβ dominant negative (IKβ DN) or IKKβ. The activity of the luciferase reporter, normalized to the co-transfected β-galactosidase, was significantly reduced ($P < 0.05$) by IKKβ overexpression, similarly to Hcy treatment. TPCK (NF-κB inhibitor) as well as IKβ DN overexpression abrogated the inhibitory effect of homocysteine on apoE promoter activity, [b]$P < 0.001$. NF-κB: Nuclear factor kappa B; TPCK: N-p-Tosyl-L-phenylalanine chloromethyl ketone; Hcy: Homocysteine; IKKβ: IκB kinase β; ApoE: Apolipoprotein E.

modulation of apoE promoter activity, we hypothesized that this downregulatory effect is exerted through NF-κB binding to the apoE promoter. Our previous data demonstrated that NF-κB p50 subunit binds to the apoE promoter[24,25]. Therefore, we tested whether NF-κB p65 subunit binds to the apoE promoter using the ChIP assay performed in HEK-293 cells treated with 500 μmol/L Hcy or cysteine (Cys). Chromatin was immunoprecipitated with anti-p65 antibodies and analyzed by PCR using primers to amplify the region -100/+4 of apoE gene. As shown in Figure 6, the results of ChIP experiments indicated that Hcy induced the recruitment of NF-κB p65 subunit to apoE promoter (Figure 6, lane "p65" Hcy). No binding of p65 proteins was observed to the apoE promoter following Cys treatment or in untreated cells (Figure 6, lanes "p65" Cys and Ctr, respectively). PCR using the input as template and primers for the apoE promoter resulted in the expected bands presented in Figure 6 (lanes "Input" Ctr, Cys and Hcy, respectively).

In summary, the results of our study clearly demonstrate that homocysteine downregulates apoE expression *via* MEK1/2 mediated activation of NF-κB. In addition, our results indicate that the activation of AP-1 or NFAT transcription factors is not involved in Hcy inhibition of apoE. Based on these findings, we propose a mechanism of homocysteine-mediated inhibition of apoE gene transcription, schematically illustrated in Figure 7.

DISCUSSION

Homocysteine (Hcy), a nonprotein amino acid resulted upon methionine demethylation is found in plasma in concentrations ranging from 3 to 15 μmol/L. In mild and intermediate HHCy, Hcy plasma levels are found in the domain of 16-100 μmol/L, while in severe HHCy, Hcy values are higher than 100 μmol/L. HHCy is an independent risk factor for cardio- and cerebro-vascular diseases, including atherosclerosis, as recently reviewed in[26], but has also implications in different disorders such as hepatic steatosis[27] and neurological disorders[28]. In addition, it was demonstrated that HHCy may aggravate kidney dysfunction[29] and is associated with impaired renal function in male patients with gout[30].

Our data demonstrate that high levels of Hcy decrease apoE expression in human embryonic kidney (HEK-293) cells (Figure 1A and B). The absence or the decreased apoE levels in different peripheral tissues leads to different pathologies. Kidney biopsies of apoE-deficient mice revealed increased mesangial cell proliferation and matrix formation, key features of the pathogenesis of renal diseases independently of hyperlipidemia[31,32]. In the atherosclerotic plaque, the macrophage-derived apoE plays important roles in the cholesterol efflux, in addition to its antioxidant and anti-inflammatory functions as reviewed in[33]. Thus, the decreased apoE expression in macrophages leads to
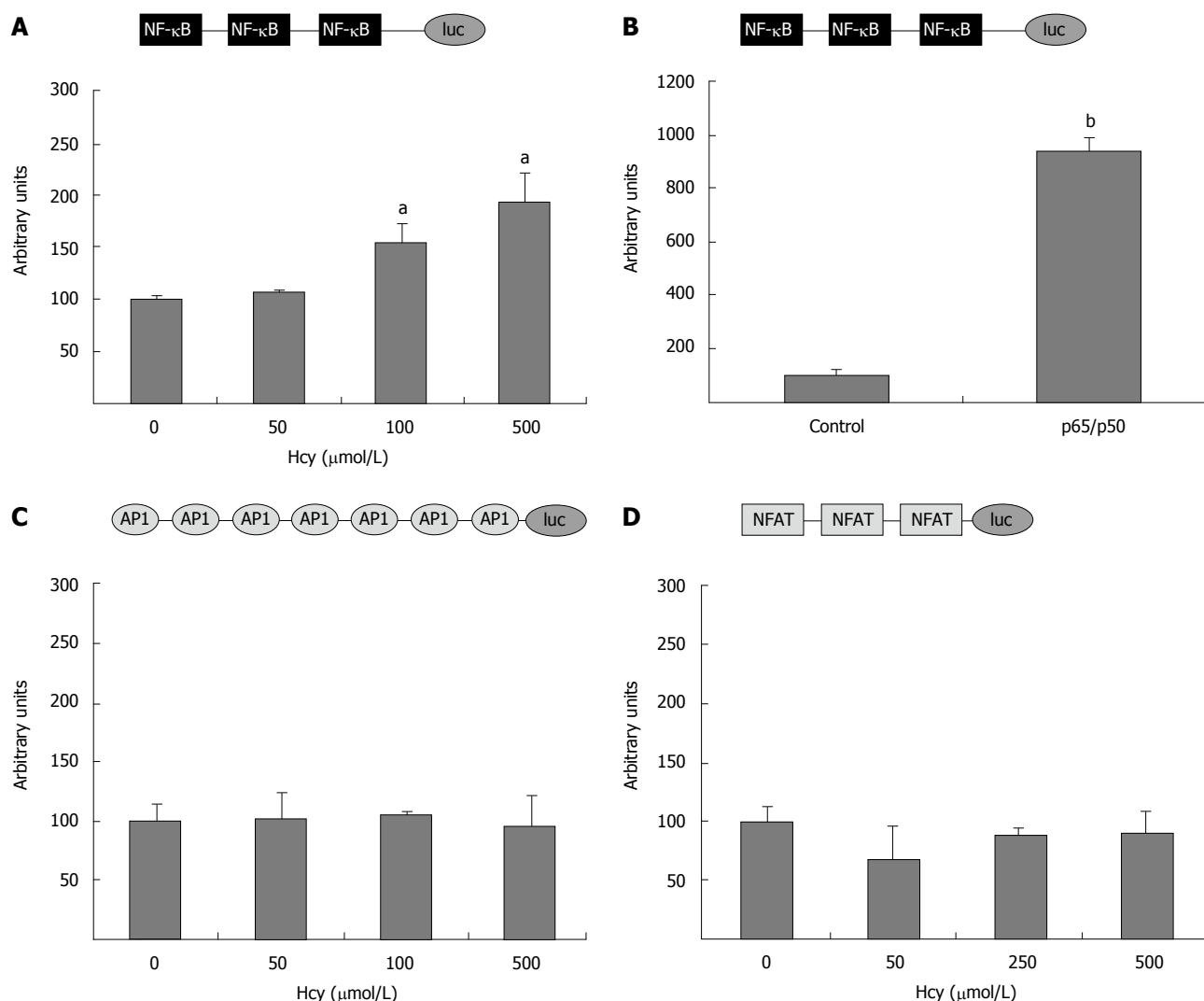
Figure 5 Homocysteine activates nuclear factor kappa B, but has no effect on activator protein-1 or nuclear factor of activated T cells activity. HEK-293 cells were transiently transfected with (NF-κB)₃-luc construct (A), (AP-1)₇-luc (C), or (NFAT)₃-luc (D) and exposed to homocysteine (50-500 μmol/L). For positive control, the cells were co-transfected with (NF-κB)₃-luc and the vector for the heterodimer p65/50. The activity of the luciferase reporter driven by the corresponding promoter was normalized to the co-transfected β-galactosidase. Treatment with high concentrations of homocysteine (250 and 500 μmol/L) significantly increased ([a]$P < 0.05$) the activity of the promoter containing the three NF-κB binding sites, while no effect of Hcy ($P > 0.05$) was observed on promoters containing binding sites for AP-1 (C) or NFAT (D). The activity of the (NF-κB)₃-luc construct was greatly enhanced by p65/p50 overexpression, used as positive control ([b]$P < 0.001$). NF-κB: Nuclear factor kappa B; AP-1: Activator protein-1; NFAT: Nuclear factor of activated T cells; Hcy: Homocysteine.

exacerbation of atherosclerosis.

Our results show that Hcy represses the endogenous apoE gene in a dose dependent manner. A significant effect on apoE expression was noticed only for Hcy doses higher than 100 μmol/L. This is relevant considering that these concentrations are similar to the severe HHCy. These doses were in the same range with those used by other authors in experiments showing the Hcy-mediated modulation of heme oxygenase-1 expression in hepatocytes[34], matrix metalloproteinase-2 in cultured rat vascular smooth muscle cells[35] or vascular smooth muscle cells proliferation[36].

To be able to exert its downstream regulatory effects, Hcy is transported into the cells as shown for primary vascular endothelial cells[37] and is transcytosed through the endothelium to reach the adjacent tissues and cells[38].

Homocysteine acts *via* NMDA receptors and renal NMDA and Group 1 metabotropic glutamate receptors have been associated with HHcy-induced glomerulosclerosis[39]. A question arises regarding the mechanism through which HCy can exert its effects in a cell line like HEK 293, which is apparently void of NMDA receptors and has been used as such to functionally characterize the activity of various receptor subtypes overexpressed in this cell line[40]. However, Hcy can exert its effects not only directly by receptor-mediated cell targeting, but also by indirect actions, as through binding to serum proteins, mainly albumin. Up to 70% of the total HCy is in its protein-bound form and there is a strong correlation between albuminemia and total HCy content[41]. Furthermore, homocysteine was found to be among the strongest protein–bound uremic toxins, which leads to reduced removal upon conventional
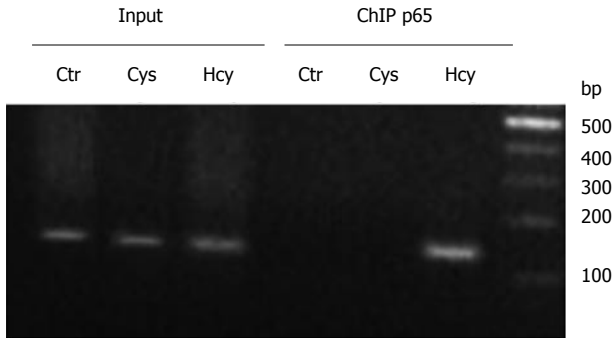
Figure 6 Nuclear factor kappa B p65 subunit is recruited to apolipoprotein E promoter following homocysteine treatment. Chromatin immunoprecipitation experiments were performed in HEK-293 cells treated with 500 μmol/L Hcy or Cys. Treatment with Hcy induced the recruitment of NF-κB p65 subunit to apoE promoter (lane p65 Hcy), while no binding of p65 proteins was observed on the apoE promoter following Cys treatment or in untreated cells (lanes p65 Cys and Ctr, respectively). PCR using the input as template and primers for the apoE promoter resulted in the expected bands (lanes Input Ctr, Cys and Hcy, respectively). NF-κB: Nuclear factor kappa B; ApoE: Apolipoprotein E; Hcy: Homocysteine; Cys: Cysteine.

hemodialysis and thus poor prognosis in atherosclerosis-associated renal complications. Thus, an indirect action could explain the high, non-physiological concentrations at which effects were noticed in our experiments, as well as in reports of other groups. Additionally, it was shown that Hcy could act as a ligand[42,43] or a competitive inhibitor[44,45] for various amino acid transport systems in both laboratory animals and humans, not only in renal cells, but also in other cell types. Therefore, despite the lack of NMDA receptors, it could be envisioned that Hcy may exert its effects in HEK-293 cells by certain non-receptor, indirect actions.

We studied the regulatory mechanisms of Hcy-induced apoE modulation. For this, we used plasmids containing the luciferase gene under apoE regulatory elements, the proximal promoter and the distal enhancer (multienhancer 2), in transient transfection experiments. The data showed that Hcy-mediated regulation involved the repression of the promoter activity (Figure 2). This Hcy-induced downregulation was observed also for other apolipoproteins such as apoAI[12]. The synergic decrease of the anti-atherogenic apolipoproteins apoAI and apoE is significant for atherosclerosis aggravation under hyperhomocysteinemic stress. The involvement of MEK1/2 in Hcy induced apoE regulation was also demonstrated (Figure 3). Similarly, MEK1/2 was shown to be involved in Hcy-induced regulation of MMP-9 in microvascular endothelial cells[46].

At the level of transcription factors, many downstream targets for Hcy were revealed to be implicated in the gene modulation of various proteins: PPARα for apoAI[12], CREB for Herp protein[47], Nrf2 for heme oxygenase-1[13]. Our study demonstrated by transient transfections and ChIP assay, that NF-κB is a downstream target of Hcy, involved in *apoE* gene regulation (Figures 4 and 6, respectively). In addition, our data showed that NF-κB binding sites included in a synthetic promoter
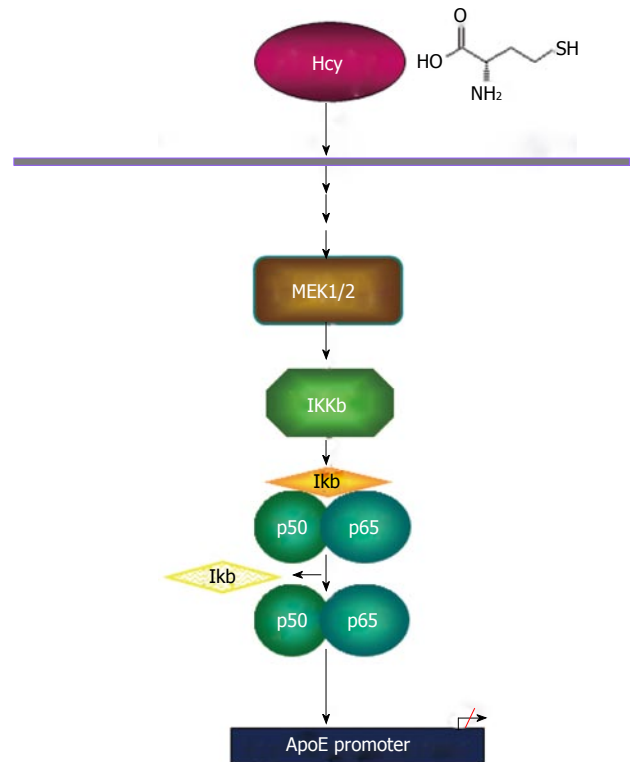

Figure 7 Schematic model of homocysteine-mediated apolipoprotein E gene downregulation. Homocysteine downregulates apoE expression *via* MEK1/2 activation, IKKβ and p65/50 subunits of NF-κB. NF-κB: Nuclear factor kappa B; apoE: Apolipoprotein E; IKKβ: IκB kinase β.

are activated by Hcy (Figure 5A). Recently, the research group of Wang demonstrated the induction in monocytes of inflammatory factors by Hcy, among which NF-κB[48]. Unexpectedly, Hcy did not activate AP-1 (Figure 5C), through MEK1/2, as we detected for LPS-induced gene modulation[24]. However, our findings are in agreement with the data concerning the involvement of NF-κB, but not AP-1, as downstream effectors of high doses of homocysteine[49]. Moreover, we also showed that NFAT pathway is not implicated in Hcy signaling (Figure 5D) in contrast with other Hcy-induced proatherogenic events modulated by this transcription factor known for its participation in pathological cardiac remodeling and vascular lesion formation[50,51].

In conclusion, we report that high level of homocysteine, through MEK1/2, activates NF-κB transcription factors that act on the apoE promoter mediating the repression of *apoE* gene expression. The importance of these findings is given by the fact that the negative effect of high Hcy concentrations on apoE expression in peripheral tissues may aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions.

COMMENTS

Background
Homocysteine (Hcy) is an independent risk factor for the cardiovascular disease and associated pathologies such as neurodegenerative diseases and renal failure. Apolipoprotein E (apoE) is an anti-atherogenic apolipoprotein

synthesized by the liver and different peripheral sources, such as macrophages, adipocytes, astrocytes, kidney cells, with an important role in lipid metabolism, atherogenesis, and inflammation. ApoE deficient mice are susceptible to atherosclerosis. Data about Hcy effects on apoE modulation are sparse, and it would be of interest to establish a possible connection between the two participants with antagonistic behavior in atherogenesis.

Research frontiers
Previous work from our and others' laboratories, demonstrated the down-regulation of apoE in macrophages under inflammatory conditions, *via* activation of the transcription factor nuclear factor kappa B (NF-κB), leading to a decreased cholesterol efflux in the atherosclerotic lesion and a consequent aggravation of the atherosclerotic phenotype.

Innovations and breakthroughs
So far, the only report connecting Hcy to apoE showed that at the post-translational level, hyperhomocysteinemia (HHCy) exerted an inhibitory effect on *in vivo* and *in vitro* dimerization of apoE directly *via* the thiol group, but no data were available on the transcriptional regulation of apoE under Hcy stress. Herein, the authors investigated the effect of increased Hcy levels on apoE expression and the mechanisms involved in this modulation. The results obtained in the present study demonstrate that Hcy inhibited apoE expression and that this negative effect is mediated *via* the activation of the pro-inflammatory transcription factor NF-κB.

Applications
The authors reported that high levels of homocysteine repress *apoE* gene expression, *via* MEK/NF-κB signaling pathway that act on apoE promoter in HEK-293 cells. This downregulation of apoE expression in peripheral tissues may be one way through high Hcy concentrations aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions and provides a link between atherogenesis, inflammation and Hcy stress.

Terminology
HHCy, characterized by an abnormally high level of plasma Hcy, is a risk factor for cardiovascular diseases.

Peer-review
In this manuscript, the authors investigated how HHCy downregulates ApoE, thus potentially contributing to the development of atherosclerosis. This is an interesting study where the experiments are reasonably well planned and executed. They collected data showing Hcy regulation of the apoE promoter and expression using cell culture model. They observed that high Hcy dose downregulated ApoE mRNA and protein in cultured RAW 264.7 and HEK293 cells and further found that activity of the ApoE proximal promoter (-500/+73)-luciferase reporter is regulated by Hcy in transient transfected cell lines. Their data potentially provide insight of Hcy related atherosclerosis and thus contribute to our understanding of atherosclerosis development.

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