NF-κB activation mechanism of 4-hydroxyhexenal via NIK/IKK and p38 MAPK pathway

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Abstract 4-Hydroxyhexenal (HHE) is known to affect redox balance during aging, included are vascular dysfunctions. To better understand vascular abnormality through the molecular alterations resulting from HHE accumulation in aging processes, we set out to determine whether up-regulation of mitogen-activated protein kinase (MAPK) by HHE is mediated through nuclear factor kappa B (NF-κB) activation in endothelial cells. HHE induced NF-κB activation by inhibitor of κB (IκB) phosphorylation via the IκB kinase (IKK)/NF-κB inducing kinase (NIK) pathway. HHE increased the activity of p38 MAPK and extracellular signal regulated kinase (ERK), but not c-jun NH_2-terminal kinase, indicating that p38 MAPK and ERK are closely involved in HHE-induced NF-κB transactivation. Pretreatment with ERK inhibitor PD98059, and p38 MAPK inhibitor SB203580, attenuated the induction of p65 translocation, IκB phosphorylation, and NF-κB luciferase activity. These findings strongly suggest that HHE induces NF-κB activation through IKK/NIK pathway and/or p38 MAPK and ERK activation associated with oxidative stress in endothelial cells.

Keywords: 4-Hydroxyhexenal; NF-κB; MAPK; IKK; NIK

1. Introduction

Lipid peroxidation induced by reactive oxygen species (ROS) is known to be involved in many processes that are biologically damaged [1,2]. Termination of lipid peroxidation occurs when two radical species react with each other to form a non-radical product [2]. Overall, lipid peroxidation is a self-propagating process that will proceed until a substrate is consumed or termination occurs.

There are broad outcomes to lipid peroxidation, structural damage to membranes and generation of bioactive secondary products fragmentation of lipid hydroperoxides, in addition to producing abnormal fatty acid esters, also liberates a number of diffusible products, some of which are potent electrophiles [3]. The most abundant diffusible products of lipid peroxidation are chemically reactive aldehydes, such as malondialdehyde (MDA), acrolein, 4-hydroxynonenal (HNE) from the ω-6 fatty acyl groups, and 4-hydroxyhexenal (HHE) from the ω-3 fatty acyl groups [1].

Reactive aldehydes from lipid peroxidation form adducts with a number of cellular nucleophiles, including protein, nucleic acids, and some lipids. HNE, especially, is a diffusible product of lipid peroxidation that has been suggested to be a key mediator of oxidative stress-induced cell death [4]. In vascular smooth muscle cells, lipid peroxidation products, such as oxidized low density lipoproteins (LDLs) or bioactive HNE promote apoptotic cell death through nuclear factor kappa B (NF-κB) activation [5]. However, some data from previous studies on reactive aldehydes are conflicting with regard to NF-κB activation [6–8]. Moreover, the mechanism by which HHE induces endothelial cell dysfunction through the regulation of NF-κB activation has not yet been well defined.

NF-κB has been shown to play a critical role in regulating the expression of large numbers of genes, including cytokines, chemokines and other mediators involved in inflammatory responses. NF-κB is a dimeric transcription factor that is composed of p50 (NF-κB1) and p65 (RelA) subunits [9]. In cells, NF-κB is retained in the cytoplasm but enters the nucleus in response to various stimuli including peroxynitrite ($ONOO^-$), nitrate (NO_2) and nitrite (NO_3) [10]. Activation of NF-κB is controlled by an inhibitory subunit, inhibitor of κB (IκB), which retains NF-κB in the cytoplasm. The activation of NF-κB requires sequential phosphorylation, ubiquitination, and degradation of IκB, as well as the consequent exposure of a nuclear localization signal on the NF-κB molecule [9].

Multiple kinases have been shown to phosphorylate IκB at specific amino-terminal serine residues [11,12]. The most studied kinases are IκB kinase, IKKα (or IKK-1) and IKKβ (or IKK-2) [13]. Strong evidence also supports the fact that IKKα and IKKβ are themselves phosphorylated and activated

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Abbreviations: HHE, 4-hydroxyhexenal; HNE, 4-hydroxynonenal; MDA, malondialdehyde; LDLs, low density lipoproteins; $ONOO^-$, peroxynitrite; NO_2, nitrate; NO_3, nitrite; NF-κB, nuclear factor kappa B; IκB, inhibitor of κB; IKK, IκB kinase; NIK, NF-κB inducing kinase; MAPK, mitogen-activated protein kinase; JNK, c-jun NH_2-terminal kinase; ERK, extracellular signal regulated kinase; MKK, MAPK kinase kinase; ROS, reactive oxygen species; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; RS, reactive species; NAC, N-acetyl cysteine

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by one or more upstream activating kinases. One such upstream kinase, NF-κB inducing kinase (NIK), was recently identified [14]. Phosphorylation of IκB by the IKK pathway eventually will lead to the nuclear translocation of NF-κB, which, in turn, will activate the expression of target genes in the nucleus [15].

Accumulated HNE caused by lipid peroxidation can induce activation and phosphorylation of extracellular signal regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPK) [16]. Two of the MAPKs, ERK and p38 MAPK, contribute to induction NF-κB activity in response to an array of extracellular stimuli [12]. Therefore, it would be interesting to know about the regulation of MAPKs during lipid peroxidation, as lipid peroxidation processes are known to enhance phosphorylation of MAPKs [17,18]. From these reports, it is possible that the activation and phosphorylation of ERK and the p38 MAPK pathway are required for HHE-induced NF-κB activation via an NF-κB translocation. Thus, in the present study, we investigated the NF-κB activation mechanism elicited by HHE induction that led to pro-inflammatory processes following NF-κB translocation and its signaling pathways.

2. Materials and methods

2.1. Cell line and culture conditions

YPEN-1, rat prostate endothelial cells, was obtained by ATCC (American type culture collection, USA). The cells were grown in DMEM (Dulbecco's Modified Eagle's Medium Media, Nissui, Tokyo, Japan) containing 2 mM L-glutamine, 100 mg/ml streptomycin, 2.5 mg/l amphotericin B and 5% heat-inactivated fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. Cells were discarded after 3 months at which time new cells were obtained from frozen stock. Cells at exponential phase were used for all experiments and cell viability (>90%) was assessed by trypan blue exclusion.

2.2. HHE treatments

Commercial HHE (purity >98%; C. NO. 32060), was obtained from Cayman Chemical Inc. Working solutions of HHE (the final concentration never exceeded the 0.1% level of ethanol) were made in PBS immediately before use. For all experiments, cells were plated in 100 mm culture dishes and cultures at 70–80% of confluences were used for the chemical exposures. After a 24 h attachment period, media were replaced with serum free media and cells were treated with 30 μM HHE after pre-incubated for 30 min with various inhibitors (ERK inhibitor, PD98059 and p38 MAPK inhibitor, SB203580). After 0–3 h, cells were harvested with ice-cold PBS. Cell lysates were used for the Western blot analysis.

2.3. Analysis of proteins by Western blot

Western blotting was carried out as described previously [19]. The cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS), and lysed in a TNN buffer (50 mM Tris–HCl, pH 8.0), 120 mM sodium chloride, and 0.5% Nonidet P-40) that was supplemented with protease and phosphatase inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 50 mM sodium fluoride), for 1 h on ice, vortexing every 5 min. Lysates were centrifuged at $9000 \times g$ for 30 min to remove insoluble material. The protein concentration was determined by the Lowry's method using bovine serum albumin (BSA) as a standard. Equal amounts of protein were separated on 10–12% SDS–PAGE gels. The gels were subsequently transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA, USA) by electroblotting for 2 h at 60–75 V. Monoclonal antibodies to p-ERK, p-p38, p-JNK, and p-IκB, and polyclonal antibodies to NF-κB (p65), IκB and p-NIK were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal antibody to p-IKKα/IKKβ was purchased from Cell Signaling Inc. (Santa Cruz, CA, USA). The membranes were blocked in a 5% non-fat milk solution in TBS with 0.5% Tween 20 and incubated with primary antibodies as indicated. Monoclonal sheep anti-mouse IgG, or donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were used at a ratio of 1:1000. Proteins were assayed by an enhanced chemiluminescence (ECL) reagent using a commercial kit (Amersham Bioscience, Bucks, UK).

2.4. Measurement of intracellular ROS levels

The cells were inoculated at a density of 3×10^4 cells/well in Costar 48 well plate, were allowed to adhere overnight and then cells were incubated in serum free DMEM with HHE and 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, USA) at 37 °C. The change in fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm by Microplate Reader FL500 (Bio-Tek Instruments, Winooski, VT, USA). A fluorometric assay was performed to determine the relative levels of ROS, such as superoxide radical, hydroxyl radical, and hydrogen peroxide [20]. This assay measures the oxidative conversion of stable, non-fluorescent DCF-DA to the highly fluorescent dichlorofluorescein (DCF) in the presence of esterases and reactive species (RS) [21]. This probe has been used extensively for quantification of ROS and lipid hydroperoxides after careful examinations for potential pitfalls in the assay conditions [22].

2.5. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) method was used to characterize the binding activities of NF-κB in nuclear extracts [23]. NF-κB oligonucleotide was 5′-GAGAGGCAAGGGGATTCCCTT-AGTTAGGA-3′. Protein-DNA binding mixture containing 20 μg of nuclear protein extract was incubated for 20 min at 4 °C in binding medium containing 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 1% NP-40, 10 mM Tris (pH 7.5), and 1 μg of poly(dI-dC)·poly(dI-dC). Radiolabeled transcription factor consensus oligonucleotide (20 000 cpm of ^{32}P) was added and the complete mixture was incubated for additional 20 min at room temperature. DNA-binding complexes were resolved by 7% native polyacrylamide gel electrophoresis with $0.5 \times$ TBE (50 mM Tris, 45 mM boric acid, and 0.5 mM EDTA) for 90 min at 200 V. The gel was dried and complexes were established with excess unlabeled oligonucleotide.

2.6. Preparation of cytosolic and nuclear extracts

Treated cells were washed, and then scraped into 1.0 ml of ice-cold PBS, and pellet at 3000 rpm at 4 °C for 5 min. The pellets were suspended in 10 mM Tris (pH 8.0), with 1.5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and protease inhibitors, incubated on ice for 15 min. Nuclei were separated from cytosol by centrifugation at 12 000 rpm at 4 °C for 15 min. The supernatants (cytosolic fraction) were removed and the pellets were suspended in 10 mM Tris (pH 8.0), with 50 mM KCl, 100 mM NaCl, and protease inhibitors, incubated on ice for 30 min, then were centrifuged at 12 000 rpm at 4 °C for 30 min.

2.7. Analysis of the intracellular $ONOO^-$ levels by confocal laser microscopy

The amount of $ONOO^-$ generated was estimated by assaying the formation of fluorescent rhodamine-1, 2, 3 from the oxidation of non-fluorescent DHR according to the modified method of McBride et al. [24]. Briefly, cells grown to subconfluence on glass coverslips were incubated with various agents for 30 min prior to treatment with HHE. Following stimulation for 3 h, cells were washed with PBS, incubated with 10 μM dihydrorhodamine 123 (DHR123) for an additional 30 min, and mounted in the microscope stage. Fluorescence images were recorded using a Zeiss LSM 510 laser scanning confocal microscope with excitation at 488 nm and long-pass detection at 530 nm.

2.8. Measurement of transfection and luciferase reporter assay for NF-κB activity

NF-κB activity was examined using a luciferase plasmid DNA, pTAL-NF-κB that contains a specific binding sequence for NF-κB (BD Biosciences Clontech, CA, USA) [25]. Transfection was carried out using FuGENE 6 Reagent (Roche, Indianapolis, IN). Briefly, 5×10^4 cells per each well were seeded in 24-well plates. When cultured cells reached about 50% confluence, cells were treated with 0.2 μg DNA/0.5 μl FuGENE 6 complexes in a total volume of normal media (5% serum contained) with 500 μl for 42 h. Subsequently, 10 μM of HHE was treated after the plate was changed with serum-free media, and treatments of PD205380 and SB203580 were performed 10 min

previously. After additional incubation for 6 h, cells were washed with PBS and added with Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) to the plate. Luciferase activity was measured by a luminometer (GENious, TECAN, Salzburg, Austria). Raw luciferase activities were normalized by protein concentration per each well.

2.9. Statistical analysis

The results were presented as means ± S.E. of three independent triplicate measurements. Statistical significance of difference between untreated control and treated groups was determined using one-way analysis of variance (post-hoc test).

3. Results

3.1. Induction of ROS and $ONOO^-$ generation by HHE

One of the products of nitrogen-derived free radicals, $ONOO^-$, is formed by the reaction of two ubiquitous free radical species: superoxide (O_2^-) and nitric oxide (NO) [21]. To verify the possible involvement of $ONOO^-$ generation in the HHE-induced apoptosis of endothelial cells, we measured intracellular ROS and $ONOO^-$ levels. Cells were treated in kinetic experiments using various incubation times and doses (0–30 μM) of HHE. The time course for intracellular ROS generation was measured by a DCF-DA method. HHE-induced ROS generation was dependent upon concentrations (Fig. 1). $ONOO^-$ levels in HHE exposed cells increased as shown in Fig. 2. At an HHE concentration of 20 μM, the $ONOO^-$ level increased significantly compared to the untreated controls. As shown in Fig. 2B, $ONOO^-$ levels were increased in HHE treated cell as compared to untreated control (see the arrow).

3.2. NF-κB activation by HHE

As shown in Fig. 3, HHE strongly induced NF-κB activation and translocation via IκBα degradation. The increase in

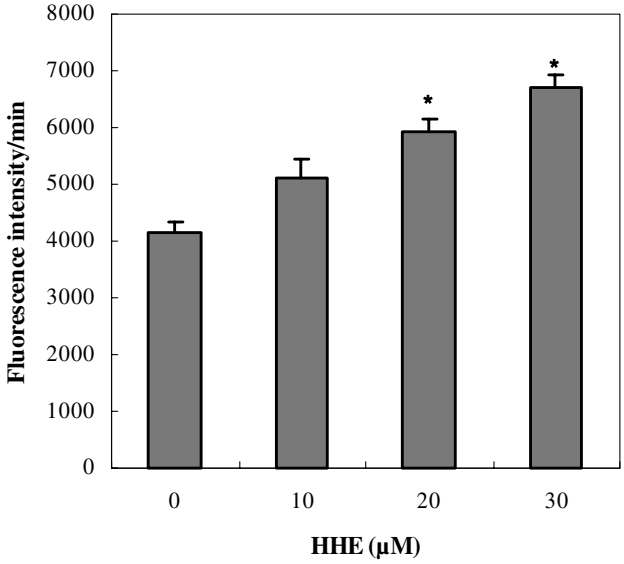

Fig. 1. ROS generation by HHE. Dose-dependent manner of intracellular ROS generation by HHE. The cells were incubated in serum free media in order to avoid the HHE binding affinity of albumin with the concentrations of HHE indicated. DCF-DA method was used to determine the intracellular ROS generation. The results are presented as means ± S.E. of three independent triplicate measurements. Statistical significance: $^*P < 0.05$ vs. untreated control.

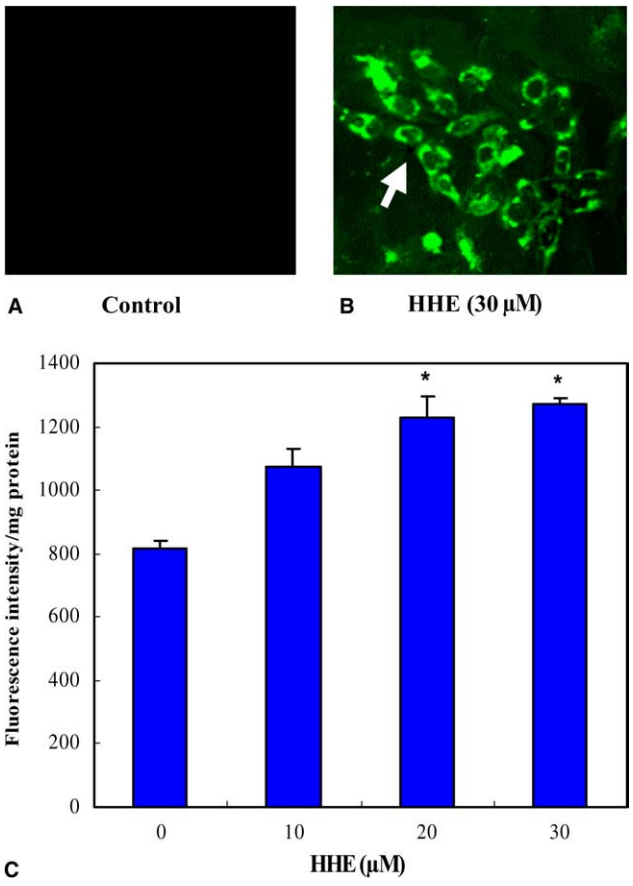

Fig. 2. Induction of $ONOO^-$ generation by HHE. Intracellular $ONOO^-$ levels were analyzed with confocal laser microscopy. (A) control; (B) 30 μM HHE; (C) induction of $ONOO^-$ generation by HHE. The cells were incubated in serum free media with the concentrations of HHE indicated for 24 h. $ONOO^-$ levels were measured by monitoring the oxidation of DHR 123 in equal amounts of cell homogenates. The results are presented as means ± S.E. of three independent triplicate measurements. Statistical significance: $^*P < 0.05$ vs. untreated control.

NF-κB activity was detectable within 1 h, continued to 2 h, and declined after 3 h of 30 μM HHE stimulation. We examined the disappearance of IκBα in cytoplasm at the same time and found that the nuclear DNA-binding activity of the transcription factor was enhanced strongly by HHE (Fig. 4). The active forms of NF-κB are heterodimers most often composed of p50 and p65. Under normal conditions, this form exists in cytosol in an inactive complex form by the inhibitory subunit IκB. Because nuclear translocation is a key step for NF-κB to exert its transcriptional activity [9], the status of the complex is crucial. Once activated, the transcription of specific sets of target genes can mediate a plethora of functions. The upregulated NF-κB activity we observed in the current experimentations is widespread biological phenomena found in aged animals [26,27] and is a critical transcription factor responsible for the pathogenesis of many disorders, including inflammatory diseases [28].

3.3. NIK–IKKα/β dependent IκBα phosphorylation induced by HHE

NF-κB is normally present in the cytoplasm in an inactive state and is bound to members of the IκB inhibitor protein

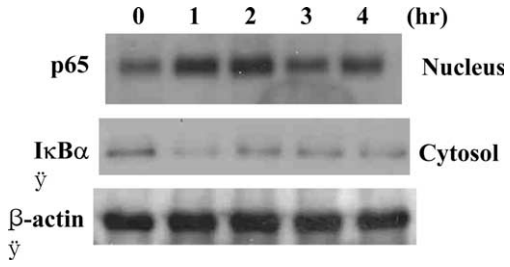

Fig. 3. NF-κB translocation and activation through IκB degradation by HHE. The cells were incubated in serum free media in order to avoid HHE binding affinity of albumin with 30 μM HHE for 0–4 h. The levels of p65 and IκBα protein in nuclear extracts (30 μg/lane) and cytosolic extracts (40 μg/lane) were analyzed by Western blot. The results are representatives of three independent experiments.

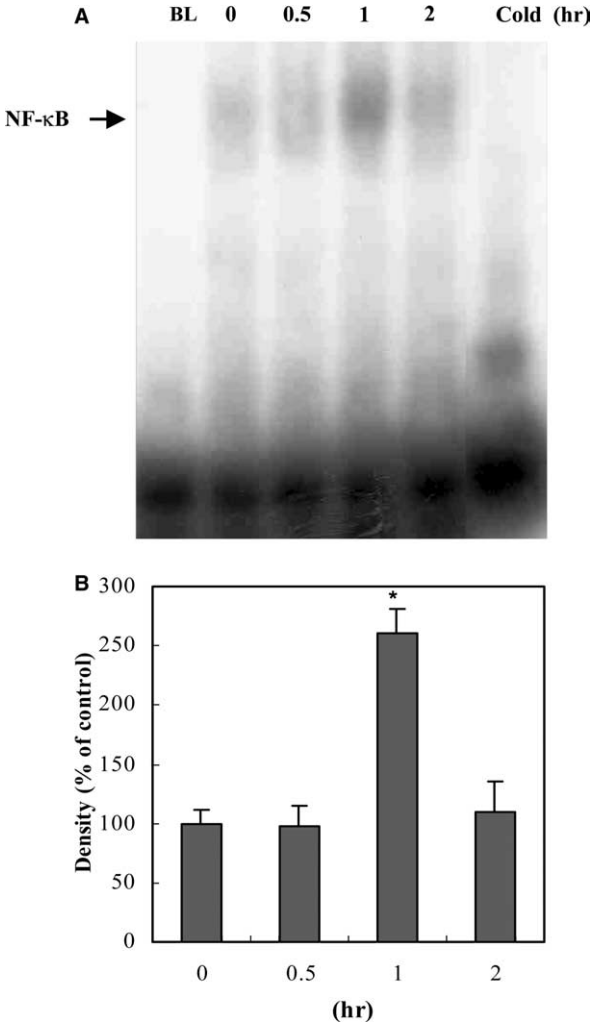

Fig. 4. Data for gel shift assay on induction of NF-κB binding activity by HHE. The cells were incubated in serum free media with HHE for the indicated times. Nuclear fractions were incubated with ^{32}P-end labeled probe containing a binding site for NF-κB (A). Gel shift assays with NF-κB probes were performed as described under Section 2. Quantification of the DNA binding activity of NF-κB was performed by densitometric analysis (B). Statistical significance: $*P < 0.05$ vs. untreated control. BL, negative control without nuclear extract; Cold, specific competition with unlabeled NF-κB oligonucleotide.

family, chiefly IκBα [9]. A common pathway, by which to achieve disruption of this complex, is based on specific phosphorylation of NIK and IKK by HHE stimuli (Fig. 5). We examined phosphorylation of NIK and IKK by Western blot analysis using anti-phospho-NIK and IKK. The increased phospho-NIK and IκBα were detectable within 1 h and continued to 3 h following 30 μM of HHE (Fig. 5).

Recently, IκBα kinase IKKα and IKKβ have been shown to phosphorylate IκB at specific amino-terminal serine residues [12]. There is strong evidence that IKKα and IKKβ are phosphorylated and activated by upstream kinase NIK [9]. Our results indicate that HHE may activate NF-κB via activation of NIK–IKKα/β-dependent IκBα phosphorylation and degradation, which, in turn, leads to NF-κB nuclear translocation.

3.4. Activation of MAP kinase by HHE

Intracellular oxidative-stress stimuli can activate both NF-κB and MAP kinase modules [29]. We examined the possibility that phosphorylation of ERK and p38 MAPK is also involved in HHE-induced NF-κB activation (Fig. 6). We measured protein levels by Western blot analysis using anti-active ERK antibody that recognizes dually phosphorylated (tyrosine and

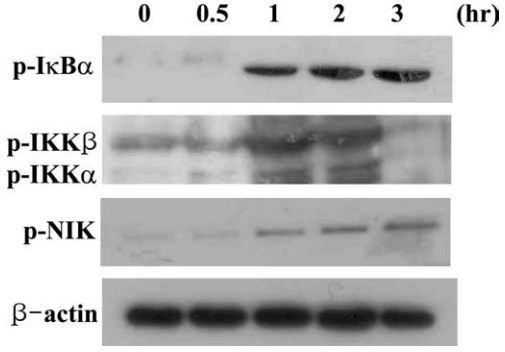

Fig. 5. Induction of the NIK and IKK activation by HHE. The cells were incubated in serum free media with 30 μM HHE for 0–3 h. The phosphorylated IκBα protein in cytosolic extracts was analyzed by Western blot with the phospho-IκBα antibody. The amount of phosphorylated proteins (IKKα/β and NIK) in whole cell was determined by Western blot analysis. The data presented are representatives of at least three separate experiments.

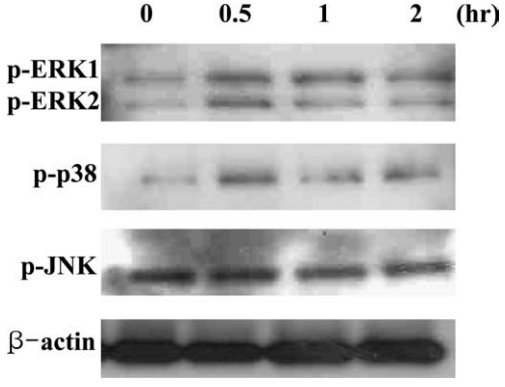

Fig. 6. Effects of HHE on the ERK, p38 MAPK, and JNK phosphorylation. The cells were incubated in serum free media with 30 μM HHE for 0–2 h. Equal amount of phosphorylated proteins determined by Western blot analysis. The data presented are representatives of at least three separate experiments.

threonine) ERK1 (44 kDa) and ERK2 (42 kDa). Results indicated that both ERK1 and ERK2 are markedly induced in the 30 μM HHE-treated cells; activated phospho-ERK1/ERK2 were detectable between 30 min and 2 h. In addition, we examined phosphorylation of p38 MAPK in YPEN cells treated with 30 μM HHE for various times. The highest level of p38 MAPK phosphorylation was observed between 30 min and 3 h. Our results also showed that the c-jun NH_2-terminal kinase (JNK) activity is not related to HHE induction.

Because nuclear translocation of NF-κB is a key step in stress induced-NF-κB activation and the activation of MAPK kinase kinase (MKK) 3/6-p38α/β MAPK pathway is required for HHE-induced NF-κB-dependent transcription, we investigated whether MKK3/6-p38α/β MAP kinase pathway mediates HHE-induced NF-κB activation via induction of NF-κB nuclear translocation [13]. Our results indicate that HHE effectively activates ERK and p38 MAPK in addition to NF-κB (Fig. 6).

3.5. Inhibitory effect of PD98059 and SB203580 on NF-κB translocation and activation

As shown in Fig. 7, the ERK and p38 MAPK inhibitors block translocation and activation of NF-κB by HHE. NIK is a member of the MAP kinase kinase kinase family that was first identified as a component of the tumor necrosis factor receptor 1 (TNF-α1)-induced NF-κB activation pathway [30]. Thus, NF-κB translocation pathway involves activation of NIK–IKKα/β, ERK and p38 MAPK. Our results clearly showed evidence that HHE activates NF-κB via NIK/IKK, p38 MAPK, and ERK dependent signaling pathways (Fig. 8).

4. Discussion

Lipid peroxidation and its associated reactive products, HHE and HNE, are known to cause age-related redox disturbances and various degenerative processes, including vascular dysfunction [31,32]. Age-related endothelial cell deteriorations due to oxidative stress are intricately involved in vascular dysfunctions. Among lipid aldehydes, HHE has strong deleterious effects because of its reactivity. However, to date, the molecular mechanism by which HHE acts on endothelial cell function has not been fully explored in regard to the intracellular signaling pathway.

New information generated from this current study on the HHE action owes to the reactive aldehydes' characteristic long

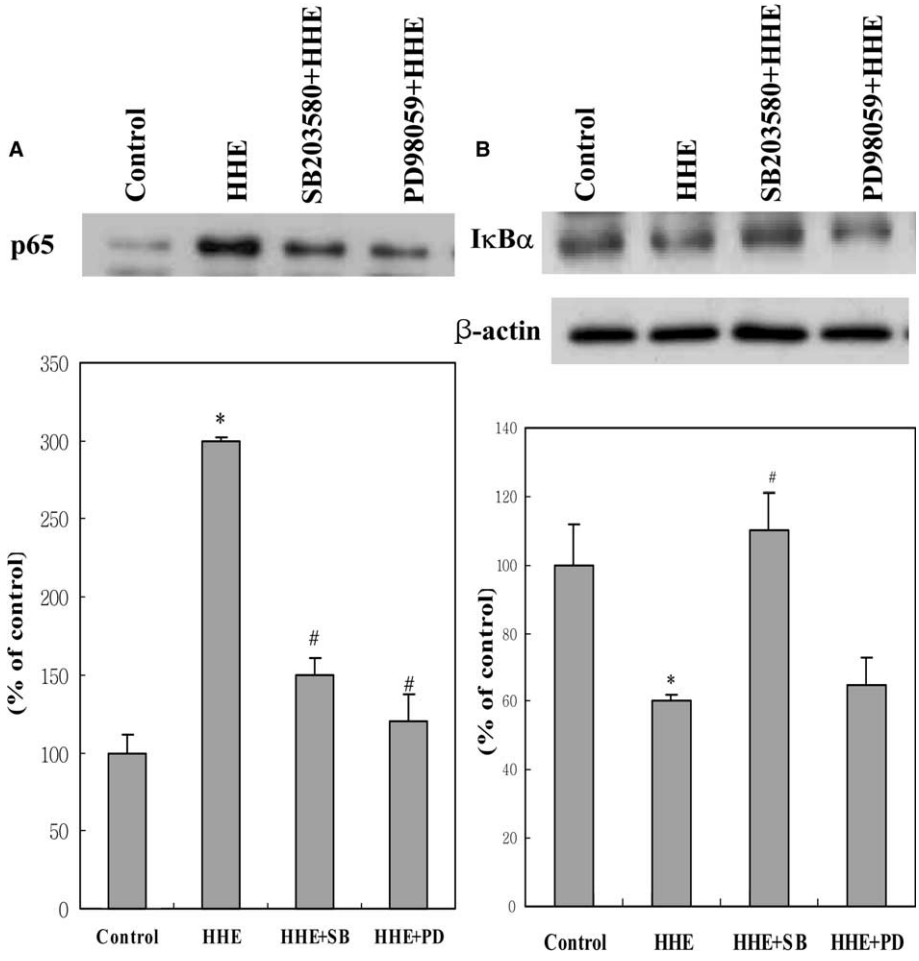

Fig. 7. Suppression of p65 translocation and IκB degradation by SB203580 and PD98059. The cells were incubated with HHE (30 μM) for 1 h after pretreatment for 30 min with SB203580 (10 μM) or PD98059. The levels of p65 (A) and IκBα (B) protein in nuclear extracts (30 μg/lane) and cytosolic extracts (40 μg/lane) were analyzed by Western blot. The data presented are representatives of at least three separate experiments. Quantitation of the p65 and IκBα expression was performed by densitometric analysis. Statistical significance: $^{*}P < 0.05$ vs. untreated control; $^{\#}P < 0.05$ vs. 30 μM HHE.

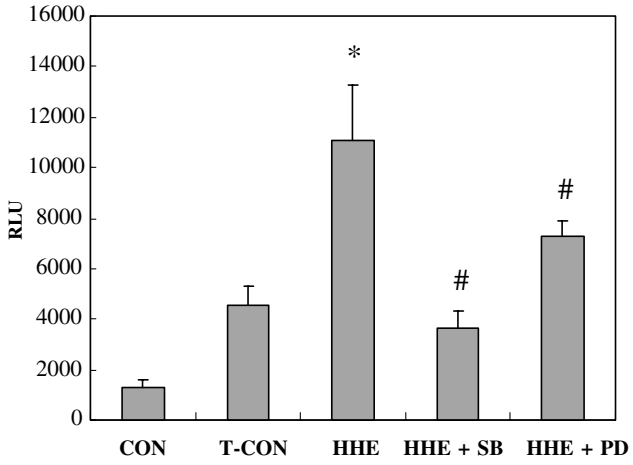

Fig. 8. Inhibitory effect of SB203580 and PD98059 on the luciferase activity of NF-κB. Cells were grown in 80–90% confluences after transfecting a reporter plasmid containing pTAL-NF-κB. The cells were co-treated with 10 μM HHE and PD98059 (10 μM) and SB203580 (10 μM) for 6 h. CON; untransfected cells, T-CON; transfected and untreated cells, HHE; transfected cells with HHE (10 μM), HHE + SB; transfected cell with HHE (10 μM) and SB203580 (10 μM), HHE + PD; transfected cells with HHE (10 μM) and PD98059 (10 μM). RLU; reactive light units. Statistical significance: $^{*}P < 0.05$ vs. untreated control; $^{\#}P < 0.05$ vs. 10 μM HHE.

half-life, easy diffusivity, and permeation through the membrane, which is in contrast to free radicals and other RS [33]. Therefore, reactive, non-charged HHE is expected to easily migrate through the cytosol from the site of production in the endothelial membranes, eliciting the diverse HHE's action on the cellular functions, including the regulation of gene transcription. In human venous plasma, normal HNE levels were estimated between 0.3 and 1 μM [34]. However, under pathological conditions, concentrations of HNE can be markedly enhanced, accumulated in cellular membranes at concentrations up to 1 mM in response to oxidative insult [1,35]. Thus, we tried to induce the NF-κB activation with 30 μM HHE in endothelial cells.

It has been reported that in vascular smooth muscle cells, lipid peroxidation products, such as oxidized LDLs or bioactive HNE, promote apoptotic cell death through NF-κB activation [5]. However, some of the data from previous studies are conflicting with regard to the precise mode of NF-κB activation [36,37]. For HHE, although HHE-induced NF-κB activation might be elicited by oxidative stress [3], the mechanism by which it regulates endothelial cell dysfunction through the regulation of NF-κB activation is not well defined.

In our study, evidence indicated that HHE exposure increased oxidative stress in endothelial cells. Among many transcription factors involved in the intracellular signaling pathway, NF-κB is known to be exquisitely sensitive to cellular oxidative status. Thus, the redox-sensitive transcription factor, NF-κB, could play a crucial role in modulating the expression of a variety of genes involved in inflammatory responses, cell adhesion, cell cycle, survival and apoptosis in multiple tissues [38–40]. Among the many well-documented examples are the dysregulation of NF-κB activation in inflammatory and vascular diseases [41,42] through the induction of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) [26,43].

Moreover, vascular changes in structure and function due to altered ECs are intricately influenced by the redox-sensitive NF-κB activation of the pro-inflammatory response. We hypothesized previously that inflammation and related pro-inflammatory processes are major underlying causes of aging-related chronic disease processes [43]. Based on the essential involvement of NF-κB in inflammatory responses, we propose that HHE induces potent inflammatory responses through the activation of NF-κB process.

The key step involved in the NF-κB regulation is the translocation of activated NF-κB from the cytoplasm to the nucleus. Cytoplasmic retention of the inactive IκB/NF-κB complex is thought to be due to the masking of the dual nuclear localization signals located on NF-κB by IκB [44]. On the basis of this recent revelation, we investigated the role of the NIK–IKK pathway in HHE-induced NF-κB activation. Our result clearly demonstrated that HHE activates NF-κB via activation of NIK–IKKα/β-dependent IκBα phosphorylation and degradation, leading to NF-κB nuclear translocation.

Recent studies reported the involvement of HNE in stress-mediated signaling pathway [45]. Several studies showed that JNK activation is critical for HNE-mediated apoptosis [16]. For instance, Tamagno et al. [46] revealed that HNE mediates amyloid β-induced neuronal apoptosis by the activation of JNK and p38 MAPK. In the present study, HHE activated p38 MAPK and ERK, which led to NF-κB activation. One possible action of p38 MAPK and ERK during NF-κB activation is the IKK phosphorylation. The other mechanism by which HHE-induced NF-κB activation is brought about is directly through p38 MAPK activation. The transactivation of NF-κB by phosphorylation and subsequent interaction with the co-activator protein CBP (CREB-binding protein) is shown to lead to both acute and chronic inflammatory responses [47]. In our study, we observed no effect of HHE on JNK activity, indicating that JNK may not be involved in HHE-induced NF-κB activation.

It has been suggested that NF-κB activation may be mediated by two distinct signaling pathways. First, the NF-κB translocation dependent pathway involves NIK–IKKα/β-dependent IκBα phosphorylation and degradation [45]. Second, phosphorylation of ERK and p38 MAPK leads to NF-κB translocation [48]. Although the effect of HNE on NF-κB activation was well studied, the HHE's modulation of NF-κB activation through the IKK/NIK pathway between the p38 sub-group of MAP kinase has not been reported.

In the present study, a specific inhibitor of MEK, PD98059 (an upstream activator of EERK1 and ERK2), or a specific inhibitor of p38, SB203580, suppressed the HHE-induced NF-κB activation. Thus, decreased NF-κB activity by these specific inhibitors strongly indicates the involvement of ERK and p38 MAPK in the HHE-induced NF-κB activation. The present study shows for the first time that HHE associated activation of ERK and p38 MAPK signaling pathways in NF-κB transactivation via IKK/NIK pathway in endothelial cells. In our previous study [49], N-acetyl cysteine (NAC), ROS inhibitor, and penicillamine, $ONOO^{-}$ scavenger, blocked RS-mediated endothelial apoptosis induced by HHE and suppressed HHE-induced ROS and $ONOO^{-}$ levels. These and current data together strongly indicate the implication of RS in HHE-induced NF-κB activation via MAPK.

Recently, a study showed that the involvement of ERK, JNK and p38 MAPK in HNE mediated actin stress fiber

formation and barrier function in lung endothelial cells [50]. Our present results strongly indicate that HHE not only increased phosphorylation of ERK and p38 MAPK, but also led to NF-κB translocation and activation via NIK/IKK pathway. This activation and phosphorylation of ERK and the p38 MAPK pathways are required for HHE-induced NF-κB activation and translocation. This interaction may be a cross-talk site between the NF-κB signaling pathways with MAPK-related other signaling pathways. Our data provide new insights into the molecular events of HHE-induced endothelial cells dysfunction and the insidious vascular inflammatory process that is cornerstones in the development of most major chronic, age-related diseases.

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