4-Hydroxy-2-hexenal-induced apoptosis in human renal proximal tubular epithelial cells

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Abstract

Background. The aldehyde products of lipid peroxidation such as 4-hydroxy-2-hexenal (HHE) might be responsible for the pathogenesis of kidney injury. The present study was aimed to investigate the effects of HHE on renal tubular epithelial cells and its signaling mechanisms.

Methods. Human proximal tubular epithelial (HK-2) cells were treated with 10 μM of HHE. Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The fluorescent probe 2′,7′-dichlorofluorescein diacetate was used to measure intracellular levels of reactive oxygen species (ROS). The protein expression of NF-κB, mitogen-activated protein kinase (MAPK), pro-apoptotic Bax and anti-apoptotic protein Bcl-2 was determined by semiquantitative immunoblotting. Apoptosis was assessed by flow cytometry analysis after the cells were stained by fluorescein isothiocyanate-conjugated annexin V protein and propidium iodine.

Results. Treatment with various doses of HHE resulted in dose-dependent decreases of cell viability and increases of ROS. HHE increased the expression of p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). HHE induced NF-κB activation and IκB degradation. Increased nuclear NF-κB activation was blocked by inhibitors of ERK (PD98059) or JNK (SP600125), but not affected by p38 MAPK inhibitor (SB203580). Flow cytometry analysis revealed HHE-induced apoptosis. HHE decreased the expression of Bcl-2, while it increased that of Bax, which was attenuated by the treatment of NF-κB inhibitor (Bay 11-7082) or N-acetyl-DL-cysteine (NAC). An inhibition of NF-κB prevented HHE-induced apoptosis.

Conclusions. HHE-induced tubular cell apoptosis is mediated by modulation of Bax and Bcl-2 via ROS generation. HHE-mediated accumulation of ROS may induce redox-sensitive transcription factor, NF-κB, through activation of ERK and JNK, resulting in cellular apoptosis in HK-2 cells.

Keywords: apoptosis; HK-2 cells; 4-hydroxy-2-hexanal; lipid peroxidation; NF-κB

Introduction

Oxidative stress has been implicated in the pathogenesis of a wide variety of human diseases. Reactive oxygen species (ROS) may elicit oxidative decomposition of polyunsaturated fatty acids (i.e. lipid peroxidation), leading to the formation of a complex mixture of aldehydic metabolites [1] and hence promoting the modification of cellular proteins [2]. In the kidney, experimental glomerulonephritis has been associated with an increased lipid peroxidation [3]. A pathological role of lipid oxidation has been also suggested in chronic kidney diseases [4]. ROS and their products may play an important role in programmed cell death [5]. Cytochrome c is a pro-apoptotic factor released from the outer surface of the inner mitochondrial membrane at early steps of apoptosis. Its release can be initiated by pro-apoptotic protein Bax and blocked by anti-apoptotic proteins Bcl-2 and Bcl-xL [6]. An increased Bax/Bcl-2 ratio induced by enhanced ROS production in tubular epithelium may be responsible for the apoptotic death in the kidney [7, 8]. The present study was aimed at further investigating the molecular mechanisms underlying the oxidative stress-induced apoptosis in tubular epithelial cells. 4-hydroxy-2-hexenal (HHE), which exhibits cytopathological effects such as mitochondrial dysfunction and apoptosis [9–11], was used as an aldehyde product of ROS.

Materials and methods

Cell culture and reagents

Human renal proximal tubular epithelial cells, HK-2 (ATCC, Manassas, VA), were cultured. Cells were passaged every 3–4 days in 100-mm dishes containing combined Dulbecco’s modified Eagle’s medium-F-12 medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma).
Effects of HHE on apoptosis in HK-2 cell

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
Viability of HK-2 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [12]. HK-2 cells were sub-cultured in 96-well plate at an initial density of 5 × 10⁴ cell/mL. Cells were incubated with fresh medium containing 0, 5, 10, 20 or 30 μM of HHE for 24 h. At the end of the experimental periods, MTT (Sigma) was added into each well with a final concentration of 0.5 mg/mL and subsequently incubated for 4 h at 37°C. Supernatants were removed by aspiration and then dimethyl sulfoxide was added to solubilize the precipitated dyes. Absorbance was measured at a wavelength of 570 nm. The viability of the cells was expressed as the fraction of surviving cells relative to untreated controls.

Intracellular level of ROS
HK-2 cells were cultured in 96-well plates until they reached confluence. Cells were incubated with fresh medium containing 0, 5, 10 or 30 μM of HHE for 24 h. The formation of ROS was detected using the ROS-sensitive fluorescent dye 2′, 7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes) for 30 min at 37°C. Fluorescence intensity was analyzed by a fluorescence reader (Fluoroscan Ascent FL; Lab systems, Helsinki, Finland) using 485 nm excitation and 538 nm emission filter. HK-2 cells were cultured on a 6-well plate for DCF-DA staining.

Nuclear extracts preparation
For nuclear extracts, cells were lysed using NE-PER® nuclear extraction reagent (NER) (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s protocol. Briefly, HK-2 cells incubated with HHE were harvested by scraping into cold phosphate-buffered saline (PBS), pH 7.2 and then centrifuged at 14 000 g for 10 min, the supernatant (nuclear extract) fraction was transferred to a clean tube [13, 14].

Western blot analysis
The cells were harvested, washed twice with ice-cold PBS and resuspended in lysis buffer (20 mM Tris–HCl, pH 7.4, 0.01 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 μg/mL leupeptin, 1 mM NaN₃VO₄) and sonicated briefly. After centrifugation, the supernatant was prepared as protein extract, and protein concentrations were measured (Pierce BCA protein assay reagent kit; Pierce Biotechnology). Equal amounts of protein were separated on 9 or 12% sodium dodecyl sulfate polyacrylamide gels. The proteins were electrophoretically transferred onto nitrocellulose membranes using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hercules, CA). The blots were blocked with 5% milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, and 0.1% Tween-20 at pH 7.5) for 1 h. The anti-extracellular signal-regulated kinases (ERK), anti-phosphorylated ERK (p-ERK), anti-Bcl-2, anti-Bax, and NF-κB p65 (Cell Signaling Technology, Beverly, MA) and β-actin (Sigma) antibodies were diluted in a blocking buffer and incubated with the blots overnight at 4°C. The bound antibodies were detected with a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibody according to the instructions provided with the ECL kit (Amersham, Franklin Lakes, NJ).

Annexin V/propidium iodide staining assay
HK-2 cells were collected at the end of treatment, washed twice with ice-cold PBS and then fixed in 70% ethanol at 4°C for 12 h. After fixation, the cells were washed twice with PBS and incubated in PBS containing propidium iodide staining (PI), RNase A, Triton X-100 (0.5%) at 37°C for 30 min. The fluorescence emitted from the propidium–DNA complex was measured using FACScan flow cytometry (Becton Dickinson, San Jose, CA). Cells containing hypodiploid DNA were considered apoptotic. The apoptosis ratio was measured using FACScan flow cytometer (Becton Dickinson) according to the instructions provided with the annexin V/PI kit. Annexin V binding and PI staining were determined by flow cytometry. After treatment with 10 μM HHE for 16 or 24 h, HK-2 cells were harvested and washed twice with pre-cooled PBS and re-suspended in a binding buffer containing fluorescein isothiocyanate (FITC)-conjugated annexin V protein and PI. After incubation in the dark for 30 min, the cells were analyzed using flow cytometry. Cells stained with both PI and annexin V were considered necrotic, and the cells stained only with annexin V were considered apoptotic [15, 16].

Statistical analysis
Results are presented as means ± SEM of three individual experiments. Differences were analyzed by analysis of variance with post hoc comparison. Statistical significance of differences was accepted at the level of P <0.05.

Results
ROS production and cytotoxicity of HHE
The formation of ROS was detected using the ROS-sensitive fluorescent dye 2′, 7′-dichlorofluorescein (DCF) in HK-2 cells. HHE treatment resulted in a dose-dependent increase of DCF fluorescence after incubation of 24 h (Figure 1). Accordingly, the treatment with HHE decreased the cell viability as determined by MTT assay (Figure 2). The HHE-induced decreased cell viability was attenuated by 1h pre-treated 10 mM NAC (data not shown).

ERK 1/2 and JNK expression
In HK-2 cells, the protein expression of p-ERK was increased after incubation of HHE (10 μM) for 1, 2 or 3 h. p-JNK expression also increased in HK-2 cells incubated with HHE (10 μM) for 30 min, 1, 2 or 3 h. p-P38 MAPK activation was apparent at 30 min, peaked at 2 h after HHE treatment (Figure 3). On the contrary, the expression of total ERK, JNK and p38 was not affected by HHE. HHE-induced increased expression of p-ERK and p-JNK in HK-2 cells was attenuated by 1h pre-treated NAC (10 mM), while p-P38 MAPK was not affected by NAC (Figure 4).

NF-κB expression
Figure 5 shows the changes of NF-κB p65 subunit levels in nuclear extracts of HK-2 cells incubated with HHE (10 μM). The expression of p65 subunits of NF-κB started to increase 1 h after HHE incubation. Cytoplasmic total IκBα expression began to decrease at 1 h, stayed decreased at 2 h and then returned to the level of before treatment.
Effects of specific chemical inhibitors, such as ERK (PD98059), JNK (SP600125) and p38 (SB203580), are shown in Figure 6. SB203580 did not have any affect, while PD98059 or SP600125 attenuated, the expression of nuclear NF-κB p65 subunit induced by HHE. These findings suggest that ERK and JNK activation is involved in the HHE-induced NF-κB nuclear translocation, while p38 MAPK pathway is not. The HHE-induced increased expression of NF-κB p65 subunit in nuclear extracts of HK-2 cells was also attenuated by 1-h pre-treated NAC (10 mM) (Figure 6).

Semiquantitative immunoblotting revealed that Bay 11-7082, NF-κB inhibitor, attenuated the HHE-induced increase of nuclear NF-κB p65 subunits, which was confirmed by electrophoretic mobility shift assay analysis (Figure 7).

Cell apoptosis

The protein expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 was measured by immunoblotting (Figure 8). An incubation with HHE for 4, 8 or 16 h at 10 μM increased ratio of Bax/Bcl-2 proteins expression. The increased ratio of Bax/Bcl-2 protein expression was counter-regulated by Bay 11-7082 or NAC (10 mM) (Figure 9). HK-2 cells treated with HHE exhibited a significant progressive increase in annexin V⁺/PI⁻ staining, which was prevented by Bay 11-7082 (Figure 10).

Discussion

The present study demonstrated that HHE treatment resulted in a dose-dependent decrease of cell viability. Accordingly, the expression of NF-κB was increased, while that of cytoplasmic total IκBα decreased. Cytoplasmic NF-κB binds to a member of inhibitory IκB protein family in an inactive state [17]. Translocation of cytosolic NF-κB into the nucleus then occurs upon phosphorylation of IκB, which is the key step for the initiation of transcription for a number of downstream proteins [17].

An imbalance between cell survival and death, a key feature of many degenerative and inflammatory diseases,
may be caused by an aberrant turnover of ROS, which regulates the cross-talk between mitogen-activated protein kinases (MAPK) and NF-κB [18]. The three major MAPK pathways terminate in the ERK, JNK/SAPK and p38, of which phosphorylation may cause NF-κB translocation [19]. The ERK pathway is activated typically by extracellular growth factors and has been linked to cell survival against apoptosis triggered by various stimuli such as oxidative stress, tumor necrosis factor-α, growth factor deprivation and apoptosis-inducing drugs [20,
Fig. 5. Expression of NF-κB p65 subunit levels in nuclear extracts of HK-2 cells incubated with HHE. The expression started to increase 1 h after HHE incubation. Cytoplasmic total IkBα expression began to decrease at 1 h and kept decreased at 2 and 4 h. *P < 0.05 versus control.

Fig. 6. Effects of PD98059 (ERK inhibition), SP600125 (JNK inhibition) and SB203580 (p38 inhibition) on NF-κB translocation (A). PD98059 and SP600125 attenuated, while SB203580 failed to affect the expression of nuclear NF-κB p65 subunit. Effects of NAC on expression of NF-κB p65 subunit in nuclear extracts of HK-2 cells incubated with HHE (B). The HHE-induced increased expression of NF-κB p65 subunit in nuclear extracts of HK-2 cells also attenuated by NAC treatment. *P < 0.05 versus control, †P < 0.05 versus HHE.

Fig. 7. Expression of nuclear NF-κB p65 subunit proteins. Semiquantitative immunoblotting revealed that Bay 11-7082 (NF-κB inhibitor) attenuated HHE-induced increase of nuclear NF-κB p65 subunits, which was confirmed by electrophoretic mobility shift assay analysis. P < 0.05 versus control, †P < 0.05 versus HHE.
Other investigations have also shown a pro-apoptotic role of ERKs in Fas- and \( \text{H}_2\text{O}_2 \)-induced apoptosis [22, 23]. The effect of ERKs on apoptosis may depend on the type of triggering and subsequent transducing signals. On the contrary, the JNK/SAPK and p38 are activated by a variety of stress factors including oxidants and inflammatory cytokines and have been linked to cell death [24].

In the present study, the expression of p-ERK, p-JNK and p38 proteins was increased by HHE, suggesting that HHE activates all three MAPKs. Furthermore, PD98059 (ERK inhibition) or SP600125 (JNK inhibition) attenuated the nuclear expression of NF-\( \kappa \)B p65, while SB203580 (p38 inhibition) was without effect. These findings suggest an involvement of ERK and JNK signaling pathways in NF-\( \kappa \)B translocation in HK-2 cells. This notion may be strengthened by the finding that NAC attenuated the activation of ERK and JNK without effects on p-P38 MAPK along with nuclear NF-\( \kappa \)B p65.

Apoptosis is the most important pathway causing programmed cell death by changing the mitochondrial membrane potential. The Bcl-2 gene family members including Bax and Bcl-2 play an essential role in regulating the changes in mitochondrial outer membrane permeability. The Bax protein initiates apoptosis upon binding to the mitochondrial outer membrane by changing the permeability and release of apoptotic proteins [25]. A balance between pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins plays a main role in commencing the apoptotic pathway. In the present study, the expression of Bax proteins was increased, while that of Bcl-2 decreased by HHE. These findings suggest that an increase of Bax/Bcl-2 ratio may have contributed to the HHE-induced apoptosis.

Apoptosis and necrosis are two distinct modes of cell death that differ in both morphology and underlying mechanisms [26, 27]. When apoptosis occurs, phosphatidylserine is translocated to the external surface of the membrane. Annexin V is a \( \text{Ca}^{2+} \)-dependent binding protein that has high affinity for phosphatidylserine. Externalization of phosphatidylserine can be assessed by measuring the extent of FITC-annexin V-binding. By contrast, membrane integrity is compromised in necrotic cell death and can be assessed using PI staining of DNA. Thus, cells marked by PI indicates a modification of cell permeability, which is a sign of necrosis. We found that HK-2 cells treated with HHE exhibited a significant progressive increase in annexin V/PI staining, indicating that HHE-induced apoptosis. Importantly, the HHE-induced increased expression of Bax proteins and decreased expression of Bcl-2 was attenuated by NAC pre-treatment. In addition, NF-\( \kappa \)B inhibitor attenuated HHE-induced changes of Bax and Bcl-2 expression and prevented HHE-induced apoptosis. The decreased apoptosis by this specific inhibitor strongly indicates an involvement of ROS-induced NF-\( \kappa \)B signaling pathways in HHE-induced apoptosis.
In summary, HHE-induced cellular apoptosis is mediated by the modulation of Bax and Bcl-2 via ROS generation in HK-2 cells. HHE-mediated accumulation of ROS may induce redox-sensitive transcription factor, NF-κB, through activation of ERK and JNK.

Acknowledgements. This study was supported by a grant from the Ministry of Science & Technology (MoST) Korea Science & Engineering Foundation (KOSEF) (2010-0021808), by the Korea Science and Engineering Foundation through the Medical Research Center for Gene Regulation grant R13-2002-013-05004-0 at Chonnam National University and by a grant (CRI10054-1) from Chonnam National University Hospital Research Institute of Clinical Medicine.

Conflict of interest statement. None declared.

References


Received for publication: 24.1.11; Accepted in revised form: 9.6.11