ISSN : 1225-9918
Journal of Life Science **2011** Vol. 21. No. 7. 961~968 DOI : 10.5352/JLS.2011.21.7.961

4-Hydroxynonenal Induces Endothelial Cell Apoptosis via ROS and Peroxynitrite Generation

Sang Woon Chung, Su Bog Yee, Ji Young Lee, Mohammad Akbar Hossain, Dong Hwan Kim, Jeong-Hyun Yoon, Hae Young Chung and Nam Deuk Kim*

Department of Pharmacy, College of Pharmacy, Pusan National University, San 30, Jangjeon-dong, Geumjung-gu, Busan 609-735, Korea

Received June 14, 2011 / Revised June 27, 2011 / Accepted June 27, 2011

The formation of reactive lipid aldehydes, 4-hydroxynonenal (HNE) is shown to be derived from fatty acid hydroperoxides through the oxidative process. Among its known effects in cytotoxicity, HNE has been implicated in apoptotic cell death. To delineate its putative role as a potential mediator, we investigated the mechanism by which HNE induces apoptosis of endothelial cells (ECs). The anti-proliferative effects of HNE were tested through MTT assay after exposure to various concentrations (5~15 μM) of HNE. We observed apoptotic bodies with propidium iodide staining, and measured the HNE induction of endothelial apoptosis by flow cytometry assay. We observed that cells exposed to HNE for 24 hr resulted in increased poly(ADP-ribose) polymerase cleavage and up-regulation of Bax. Data on the HNE action strongly indicated the involvement of reactive species, namely, intracellular ROS, nitrite, and peroxynitrite. To obtain evidence on the implication of ROS and peroxynitrite in HNE-induced apoptosis, a ROS scavenger, *N*-acetylcysteine (NAC), and a peroxynitrite scavenger, penicillamine, were tested. Results clearly indicate that the induction of apoptosis by HNE was effectively inhibited by NAC and penicillamine. Based on the present data, we conclude that the endothelial apoptosis induced by HNE involves both ROS generation and peroxynitrite activity. Our new data could lead to a redefinition of HNE action on apoptosis in ECs.

Key words : 4-Hydroxynonenal, apoptosis, ROS, peroxynitrite, endothelial cells

Introduction

In recent years, many studies presented evidence pointing to the involvement of reactive aldehydes and other lipid peroxidative byproducts in various pathophysiological processes, including aging, diabetes mellitus, atherosclerosis, and cancer [16,29]. Among the major reactive aldehydes, 4-hydroxynonenal (HNE) derived from lipid peroxidation process from arachidonic acids, linoleic acids, or their hydroperoxides can be accumulated in relatively large amounts under oxidatively stressed conditions, namely it has shown to be largely responsible for the cytotoxic effects associated with oxidative stress [8,32], as detected in several diseases, such as, atherosclerosis, diabetes, and Parkinson's disease [17,35]. The formation of HNE and HNE-protein conjugates could be important biological deleterious instigators, as oxidative stress-induced apoptosis is believed to be involved in those chronic diseases. In fact, HNE has been shown to be a potential inducer of apoptotic cell death [23].

***Corresponding author**
Tel : +82-51-510- 2801, Fax : +82-51-513-6754
E-mail : nadkim@pusan.ac.kr

Apoptosis, a form of cell death characterized by cell shrinkage, chromatin condensation, and DNA fragmentation, can be induced by a variety of stimuli. The role of radicals in apoptosis become apparent with the following evidence: (a) reactive intermediates influence the cellular redox status [3], therefore, apoptosis [27], and (b) antioxidants can attenuate apoptosis [2,28]. Based on the reactivity of reactive oxygen species (ROS) on the modification of various intracellular molecules, it was previously reported that ROS induced apoptosis in a concentration-dependent manner [22]. In addition, changes in the redox status by oxidative stress modulate subsequent effects on specific downstream kinases, phosphatases, and transcription factors, which can alter the cell's sensitivity to apoptotic stimuli [1].

Another major oxidant derived form nitric oxide (NO) is peroxynitrite. NO synthesized with inducible NO synthase (iNOS) plays a key role in the host's defense mechanism, however, is also known to cause tissue damage [6]. Some of the cytotoxic effects of NO are related to the production of peroxynitrite formed during the rapid reaction of NO and superoxide [4]. The formation of peroxynitrite from the interaction between NO with the superoxide radical anion ($\bullet O_2^-$) is at nearly the diffusion limit rate [14]. Peroxynitrite is a

powerful oxidant and nitrating agent capable of modifying lipids [24], proteins [15], and DNA [7]. Indeed, peroxynitrite was known to display an important role affecting cell metabolism and signaling pathway [18]. One earlier study reported that treating endothelial cells with increasing concentrations of nitric oxide induced apoptosis by peroxynitrite [33].

Regarding the HNE effect on apoptosis, many recent studies suggest that HNE induces apoptosis in many different cell types [5,30]. *In vitro* studies have revealed that at relatively high concentrations (~50 μM), HNE caused rapid cell death associated with the depletion of sulfhydryl groups and the inhibition of key metabolic enzymes [8]. HNE induces cell growth and DNA synthesis at low concentrations, thus preventing NO production [11,26]. Although many studies have reported that HNE induced apoptosis, the precise mechanism underpinning HNE-induced apoptosis of endothelial cells through ROS and peroxynitrite generations has not been thoroughly investigated. In this study, we attempted to define the mode of HNE-induced apoptosis of endothelial cells by showing the involvement of ROS and peroxynitrite. The data also showed two potent scavengers – *N*-acetylcysteine (NAC) and penicillamine – effectively blunting and protecting endothelial cells from HNE-induced apoptosis.

Materials and Methods

Cells and cell culture conditions

YPEN-1 rat prostate endothelial cells (ECs) were obtained from ATCC (American Type Culture Collection, Manassa, VA, USA). The cells were grown in Dulbecco's Modified Eagle Medium Media (DMEM) with 5% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA) in a humidified 5% CO_2 at 37°C containing 100 U/ml penicillin, 100 mg/ml streptomycin (GIBCO BRL), and 2.5 mg/l amphotericin B (Sigma-Aldrich, St. Louis, MO, USA).

MTT assay for cell viability

The proliferation of cells was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme as previously described [31].

Flow cytometry analysis

The treated cells were trypsinized, and fixed in 95% ethanol at 4°C for 40 min. Prior to analysis, cells were again washed with PBS, suspended in cold propidium iodide (PI, Sigma-Aldrich) solution, and incubated in the dark for 30 min. Flow cytometry analyses were performed by a flow cytometry system (Becton Dickinson, San Jose, CA, USA).

Staining and nuclear structure examination

Cells were incubated with 5~15 μM HNE (Cayman, Ann Arbor, MI, USA) for 24 hr. Then cells were washed with PBS containing 1% bovine serum albumin (PBS-B) and fixed with 70% ethanol containing 0.5% Tween 20 at 4°C for 30 min. Fixed cells were washed with PBS-B, and stained with PI solution (50 μg/ml in PBS) for 30 min. The stained cells were washed twice with PBS-B and observed using a fluorescence microscope at ×320 magnification.

Protein measurement by Western blotting

Cells were harvested and washed twice in PBS at 4°C. Total cell lysates were lysed in lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 100 μg/ml phenymethylsulfonyl fluoride (PMSF)]. The supernatant was collected and protein concentrations were then measured with protein assay reagents (Pierce, Rockford, IL, USA). Equal amounts of proteins were boiled for 2 min and chilled on ice, subjected to 8-12% SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane. Monoclonal antibodies to Bcl-2 and polyclonal antibodies to Bax and poly(ADP-ribose) polymerase (PARP) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Monoclonal antibody to β-actin (Sigma-Aldrich) was used as an internal control. Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Life Science (Arlington, IL, USA). The proteins were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham Life Science).

Measurement of intracellular ROS

2,7-Dichlorodihydrofluorescin-diacetate (H_2DCFDA) was oxidized to fluorescent 2,7-dichlorofluorescin (DCF) by ROS [20]. A stock solution of 12.5 mM H_2DCFDA in 100% EtOH was stored at -20°C. Working solution with 25 μM H_2DCFDA diluted from the stock solution was placed on ice in the dark immediately prior to the study. The fluorescence intensity of DCF was measured by using a micro-

plate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, Winooski, VT, USA) with excitation and emission wavelengths of 485 nm and 530 nm.

Quantification of NO_2^-/NO_3^- and peroxynitrite

Resulting NO_2^- was mixed with a Griess reagent [10] to form a purple azo dye. The absorbance of the product dye at 540 nm was measured by a flow-through spectrophotometer. However, NO_3^- became NO_2^- in the presence of NO_2^- reductase and NADPH in Griess assay. The absorbance of the product dye at 540 nm was measured with a microplate reader [10].

Peroxynitrite activity was measured by monitoring the oxidation of DHR 123 by modifying the methods of Kooy *et al.* [18]. A stock solution of 5 mM DHR 123 in dimethylformamide was purged with nitrogen and stored at -20°C. The working solution with 5 μM DHR 123 diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride was purged with nitrogen and placed on ice before use. Just before use, 100 μM diethylenetriaminepenta acetic acid (DTPA) was added. Peroxynitrite activity by the oxidation of DHR 123 was measured on a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments) with excitation and emission wavelengths of 485 nm and 530 nm at room temperature, respectively.

Statistical analysis

Values are expressed as the mean±SE. The statistical significance of the differences between the treatments was determined by the ANOVA test. Differences in the means between individual groups were assessed by Fischer's Protected LSD post-hoc test. Values of $p<0.05$, $p<0.01$, and $p<0.001$ were considered statistically significant.

Results

Cytotoxic effects and HNE-induced apoptosis on ECs

To investigate the cytotoxic effect of HNE on the viability of ECs, the MTT assay was performed using various HNE concentrations. The cells were treated with HNE at concentrations of 5, 10, 15, and 20 μM for 24 hr. HNE showed cytotoxic effects in a concentration-dependent manner. The IC_{50} value of HNE was approximately 8 μM (Fig. 1). Microphotographs of HNE-treated ECs also showed distinct morphological changes and cell viability (Fig. 2). To determine whether HNE induces apoptosis of EC, we used flow cytometry to analyze cell cycle modulation in determining whether HNE caused decreased endothelial viability (Fig. 3). Cells were accumulated at G2/M phase of cell cycle at low concentrations of HNE treatments (Fig. 3A-C). An augmentation in the sub-G1 phase of the cell cycle was observed at high concentrations of HNE treatments (Fig. 3C-D). Next, we examined structural changes in the nuclei after PI staining on HNE-treated cells. Greater nuclear fragmentation and condensation were observed on cells treated with 10 μM

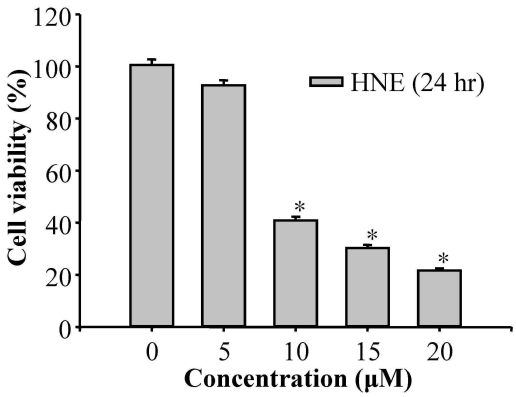

Fig. 1. Cytotoxic effect of HNE on ECs. Cells were treated with various concentrations of HNE for 24 hr, and then the percentage of cell survival was determined using the MTT assay. Result is expressed as percentage of the vehicle treated control ± SE of three separate experiments. The significance was determined by the ANOVA test (*$p<0.05$ vs. vehicle-treated control)

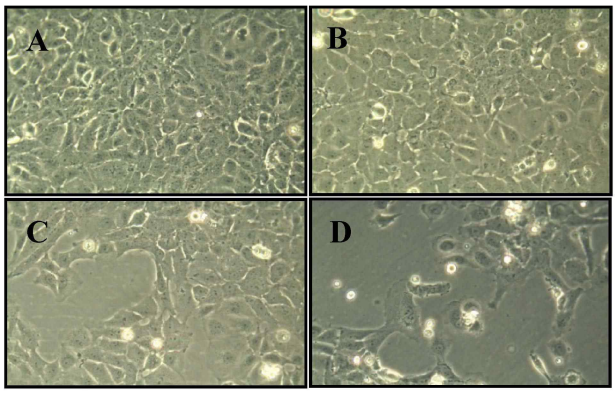

Fig. 2. Representative microphotographs of HNE-treated ECs. Cells were plated at 2×10^4 cells per 60-mm plates, incubated for 24 hr and treated with HNE for 24 hr. (A) control, (B) 5 μM, (C), 10 μM, and (D) 15 μM. The cells were photographed by phase contrast microscopy (original magnification 320×).

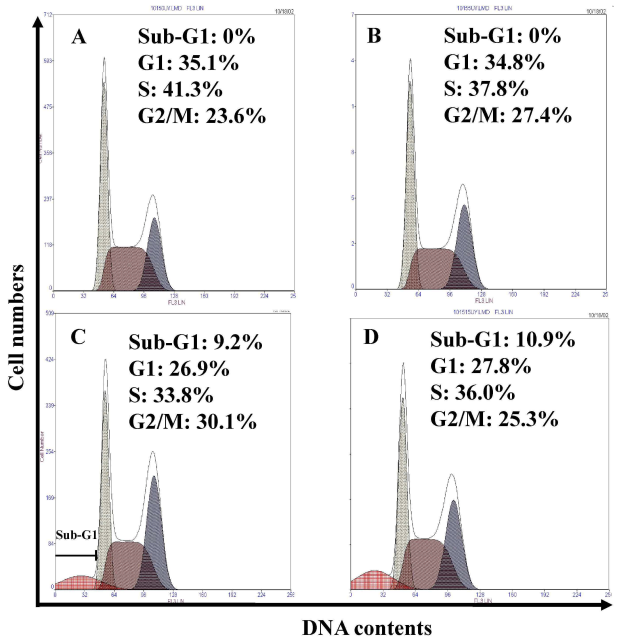

Fig. 3. Increased apoptosis-related signals after treatment with HNE. Cells were incubated with HNE for 24 hr, and analyzed flow cytometry. (A) control, (B) 5 μM, (C), 10 μM, and (D) 15 μM. DNA contents were analyzed by flow cytometry with PI staining. The cell cycle distribution was calculated as the percentage of cells contained in each phase with the CellQuest software (Becton Dickinson Instruments). All experiments were performed in triplicate and gave similar results.

HNE (data not shown). These results show that HNE causes apoptosis on endothelial cells at a concentration of 10 μM HNE.

It was reported that the Bcl-2 family is a major regulator of apoptosis and that the Bcl-2/Bax ratio appears to be an important determinant in the regulation of apoptosis [34]. To investigate the involvement of the Bcl-2 family, Bcl-2 and Bax expression levels were measured by Western blotting. The basal expression levels of Bcl-2 were down-regulated, whereas expression of Bax was up-regulated by HNE treatment (Fig. 4). Also, PARP (116 kD) was significantly cleaved into the 89 kD fragment (Fig. 4). These results suggest that HNE treatment plays a key role in inducing apoptosis, through up-regulation of Bax expression and cleavage of PARP of ECs.

Augmentation of ROS, nitrite, and peroxynitrite generation through HNE treatment

ROS reportedly plays a key role in inducing cellular apoptosis [13], and contributes to cellular injury on treated HNE.

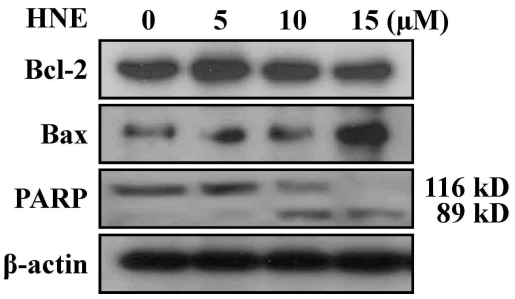

Fig. 4. Expressions of Bcl-2, Bax, and PARP proteins in ECs treated with HNE. Cells were incubated with HNE for 24 hr and total cell lysates were prepared and immunoblotted with antibodies against Bcl-2, Bax, PARP, and β-actin, and ECL detection. A representative blot is shown from three independent experiments.

The HNE-treated group produced significantly higher ROS levels compared to the untreated group in the formation of the fluorescence of DCFDA (Fig. 5A). This result suggests that HNE contributes to increase of intracellular ROS in ECs.

To determine the effects of increased NO production, the amount of nitrite accumulated in the culture medium was measured (Fig. 5B). Results showed that the levels of nitrite were increased in a dose-dependent manner. Part of the cytotoxic effects of NO are related to the production of peroxynitrite, a more potent oxidant and cytotoxic agent, which is formed by the rapid reaction between NO and superoxide [4]. As shown in Fig. 5C, it showed the amount of peroxynitrite was increased as the level of nitrite was increased. In the previous experiments, we found that HNE-induced apoptosis was followed by increased levels of ROS, nitrite and peroxynitrite. As Fig. 5 indicates, HNE produced ROS, nitrite, and peroxynitrite in a concentration-dependent manner to correlate with apoptotic activity.

Inhibition of HNE-induced apoptosis by NAC and penicillamine

ROS-induced oxidative stress is a common mediator of apoptosis. The ability of ROS-mediated oxidative stress to provoke apoptosis as a result of massive cellular damage has been associated with the depletions of cellular antioxidants, such as GSH, lipid peroxidation, and alterations in proteins and nuclei [25]. In this study, in order to demonstrate whether ROS and peroxynitrite are involved in HNE-induced apoptosis, an ROS scavenger NAC, and a peroxynitrite scavenger penicillamine, were used. Prior treatment of cells with 250 μM NAC or 250 μM penicillamine

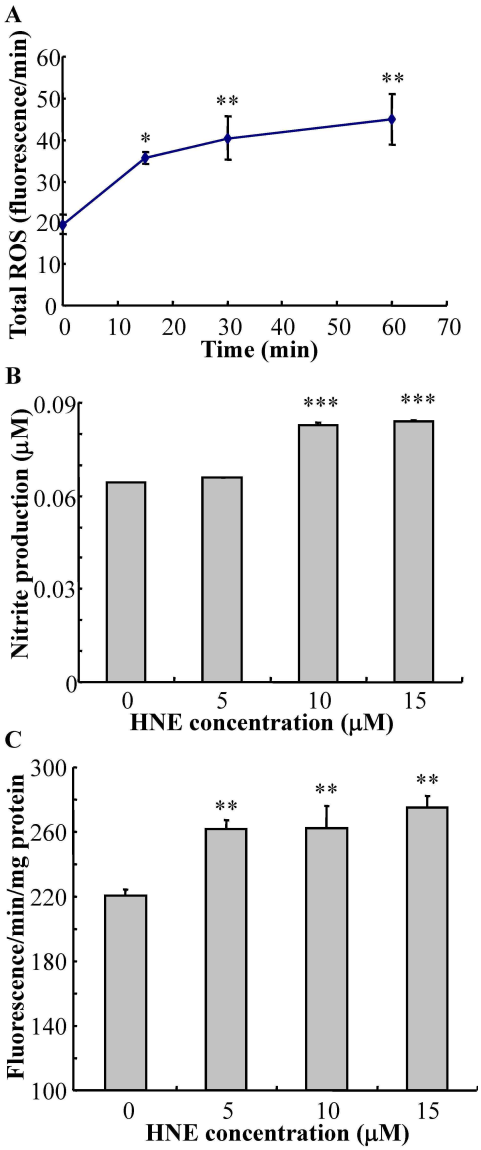

Fig. 5. Induction of ROS, nitrite, and peroxynitrite production by HNE. (A) Cells were treated with 15 μM HNE at various time. Intracellular ROS levels were measured as described in Materials and Methods. HNE treatment increased ROS generation in a time-dependent manner. (B) Cells were treated with 15 μM HNE for 24 hr, after which nitrite accumulation in the culture medium was measured as described in Materials and Methods. (C) Cells were treated with 15 μM HNE for 24 hr, after which peroxynitrite production was measured by monitoring the oxidation of DHR 123 as described in Materials and Methods. The results are resented as means±SE of three independent experiments. The significance was determined by ANOVA test (*p<0.05, **p<0.01, and ***p<0.001 vs. vehicle-treated control).

for 1 hr was followed by a HNE treatment for an additional 24 hr. The MTT assay showed significant potentiation of

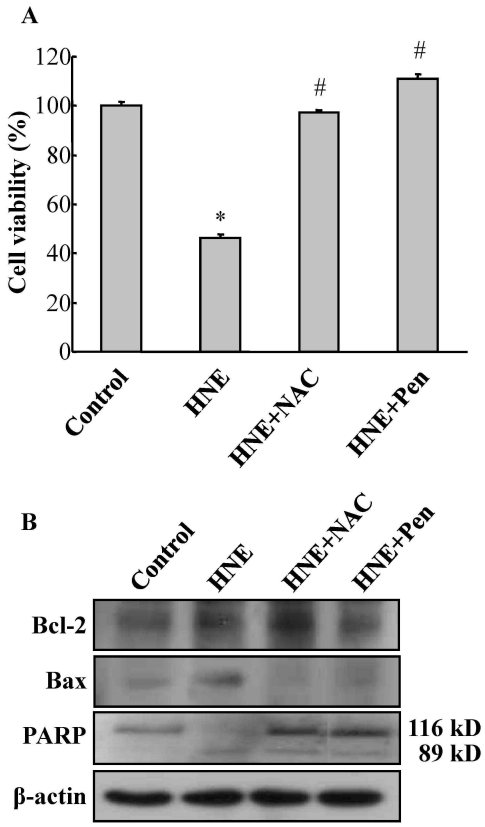

Fig. 6. Inhibition of HNE-induced apoptosis by NAC and penicillamine. Cell viability (A) and expression levels of Bcl-2, Bax, and PARP proteins (B) were determined in HNE-treated cells as described in figure legends of Figs. 1 and 4. (A) Cells were incubated with 15 μM HNE for 24 hr after pre-treatment with 250 μM NAC or 250 μM penicillamine for 1 hr and examined by MTT assay. The results are represented as means±SE of three independent experiments. The significance was determined by ANOVA test (*p<0.05 vs. vehicle-treated control; $^{\#}p$<0.05 vs. 15 μM HNE). (B) Total cell lysates were prepared and immunoblotted with antibodies against Bcl-2, Bax, PARP, and β-actin, and ECL detection. A representative blot is shown from three independent experiments. NAC, *N*-acetyl cysteine; Pen, penicillamine.

HNE-induced cytotoxicity.

Results in Fig. 6A shows that in the treatment of ECs with anti-oxidants, the cells were able to recover from the suppressed viability induced by HNE. Using Western blotting, NAC and penicillamine showed protection of ECs from HNE-induced apoptosis. The data also showed that the up-regulation of Bax and PARP cleavage was inhibited by the treatments of both anti-oxidants (Fig. 6B). Therefore, these data clearly indicate that the implication of ROS and

peroxynitrite in the induction of apoptosis by HNE.

Discussion

Much of the research demonstrates that HNE, a reactive byproduct of lipid peroxidation, plays a key role in oxidative stress induced apoptosis. Although the involvement of HNE in the induction of apoptosis is documented on ECs [12], we wanted to establish whether HNE induced apoptosis of ECs implicates ROS and peroxynitrite. Our results from this study led to a conclusion that this is most likely.

Apoptosis as determined by flow cytometry analysis was detected when ECs were treated with HNE at concentrations over 10 μM for 24 hr. Nuclear fragmentation and condensation were visible with PI staining. To confirm the quantitative data, Bcl-2 and Bax protein were examined using Western blotting because the Bcl-2 family proteins have been reported as major regulators for apoptosis. In this study, we found that the expression of pro-apoptotic Bax was up-regulated, while the anti-apoptotic protein, Bcl-2 was down-regulated, in contrast to the control group. Also, the PARP protein (116 kD) was significantly cleaved to the 89 kD fragment. The data indicated that HNE induced apoptosis on ECs through alterations in the Bcl-2 family proteins.

The redox status of cells modulated by various sources including reactive species, oxidants, and lipid peroxides that are generated as a consequence of aerobic metabolism is a prime determinant for the cellular homeostasis [21]. Thus redox imbalance has a great impact on the cell signaling pathways by activating gene expression of many redox-sensitive transcription factors, such as NF-κB, which consecutively activate apoptosis or necrosis [13]. Although ROS has been reported to play a substantial role in apoptosis [9,19], their involvements in the HNE induced apoptotic activity has yet to be documented.

Evidence we obtained from the current study strongly suggests that in the absence of reactive species, the apoptotic potency of HNE diminishes greatly. To validate the findings, scavengers, NAC and penicillamine were used in our study. We found that they prevented the activation of Bax and PARP expression and blocked apoptosis. Moreover, NAC and penicillamine blocked nuclear condensation (data not shown) and blocked expression of apoptosis signals, suggesting that HNE produces ROS and peroxynitrite for in endothelial cell death.

If our observation can be measured in other cell systems, it requires a re-evaluation of the putative mechanisms so far outlined in HNE-induced apoptosis. Further study is needed to investigate HNE-inducted apoptosis in the presence of anti-oxidants. In conclusion, this present study demonstrated that for HNE-induced apoptosis of ECs, ROS and peroxynitrite may play a substantial role. Further investigation into the involvement of reactive aldehydes in cell death at the molecular levels in the presence of anti-oxidants should provide a clearer picture on the specific mode of apoptotic reactive aldehydes' action.

Acknowledgement

This work was supported by a 2-Year Research Grant of Pusan National University.

References

1. Akgul, C., D. A. Moulding, and S. W. Edwards. 2001. Molecular control of neutrophil apoptosis. *FEBS Lett.* **487**, 318-322.
2. Banzet, N., D. Francois, and B. S. Polla. 1999. Tobacco smoke induces mitochondrial depolarization along with cell death: effects of antioxidants. *Redox Rep.* **4**, 229-236.
3. Beckman, K. B. and B. N. Ames. 1998. The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581.
4. Beckman, J. S. and W. H. Koppenol. 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* **271**, C1424-1437.
5. Camandola, S., G. Poli, and M. P. Mattson. 2000. The lipid peroxidation product 4-hydroxy-2,3-nonenal increases AP-1-binding activity through caspase activation in neurons. *J. Neurochem.* **74**, 159-168.
6. Di Rosa, M., A. Ialenti, A. Ianaro, and L. Sautebin. 1996. Interaction between nitric oxide and cyclooxygenase pathways. *Prostaglandins Leukot. Essent. Fatty Acids* **54**, 229-238.
7. Douki, T. and J. Cadet. 1996. Peroxynitrite mediated oxidation of purine bases of nucleosides and isolated DNA. *Free Radic. Res.* **24**, 369-380.
8. Esterbauer, H., R. J. Schaur, and H. Zollner. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11**, 81-128.
9. Ferrari, G., C. Y. Yan, and L. A. 1995. Greene. N-acetylcysteine (D- and L-stereoisomers) prevents apoptotic death of neuronal cells. *J. Neurosci.* **15**, 2857-2866.
10. Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* **126**, 131-138.
11. Hattori, Y., S. Hattori, and K. Kasai. 2001. 4-Hydroxynonenal prevents NO production in vascular smooth muscle cells

by inhibitions nuclear factor-κB-dependent transcriptional activation of inducible NO synthase. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1179-1183.
12. Herbst, U., M. Toborek, S. Kaiser, M. P. Mattson, and B. Hennig. 1999. 4-Hydroxynonenal induces dysfunction and apoptosis of cultured endothelial cells. *J. Cell Physiol.* **181**, 295-303.
13. Herrera, B., A. M. Alvarez, A. Sánchez, M. Fernández, C. Roncero, M. Benito, and I. Fabregat. 2001. Reactive oxygen species (ROS) mediates the mitochondrial-dependent apoptosis induced by transforming growth factor β in fetal hepatocytes. *FASEB J.* **15**, 741-751.
14. Huie, R. E. and S. Padmaja. 1993. The reaction of NO with superoxide. *Free Radic. Res. Commun.* **18**, 195-199.
15. Ischiropoulos, H. and A. B. Al-Mehdi. 1995. Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett.* **364**, 279-282.
16. Jener, P. 1994. Oxidative damage in neurodegenerative disease. *Lancet* **344**, 796-798.
17. Jürgens, G., Q. Chen, H. Esterbauer, S. Mair, G. Ledinski, and H. P. Dinges. 1993. Immunostaining of human autopsy aortas with antibodies to modified apolipoprotein B and apoprotein(a). *Arterioscler. Thromb.* **13**, 1689-1699.
18. Kooy, N. W., J. A. Royall, H. Ischiropoulos, and J. S. Beckman. 1994. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic. Biol. Med.* **16**, 149-156.
19. Kruman, I., A. J. Bruce-Keller, D. Bredesen, G. Waeg, and M. P. Mattson. 1997. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.* **17**, 5089-5100.
20. LeBel, C. P., H. Ischiropoulos, and S. C. Bondy. 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **5**, 227-231.
21. Lee, J. Y., J. H. Je, D. H. Kim, S. W. Chung, Y. Zou, N. D. Kim, M. A. Yoo, H. S. Baik, B. P. Yu, and H. Y. Chung. 2004. Induction of endothelial apoptosis by 4-hydroxhexenal. *Eur. J. Biochem.* **271**, 1339-1347.
22. Liu, S. L., D. Y. Shi, X. H. Pan, and Z. H. Shen. 2001. Inhibition of proliferation and expression of N-ras in hepatoma cells by antioxidation treatment. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* **33**, 463-466.
23. Liu, W., M. Kato, A. A. Akhand, A. Hayakawa, H. Suzuki, T. Miyata, K. Kurokawa, Y. Hotta, N. Ishikawa, and I. Nakashima. 2000. 4-Hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. *J. Cell Sci.* **113**, 635-641.
24. O'Donnell, V. B., J. P. Eiserich, P. H. Chumley, M. J. Jablonsky, N. R. Krishna, M. Kirk, S. Barnes, V. M. Darley-Usmar, and B. A. Freeman. 1999. Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **12**, 83-92.
25. Pae, H. O., G. S. Oh, B. M. Choi, E. A. Seo, H. Oh, M. K. Shin, T. H. Kim, T, O. Kwon, and H. T. Chung. 2003. Induction of apoptosis by 4-acetyl-12,13-epoxyl-9-trichothecene-3,15-diol from *Isaria japonica* Yasuda through intracellular reactive oxygen species formation and caspase-3 activation in human leukemia HL-60 cells. *Toxicol. In Vitro* **17**, 49-57.
26. Ruef, J., G. N. Rao, F. Li, C. Bode, C. Patterson, A. Bhatnagar, and M. S. Runge. 1998. Induction of rat aortic smooth muscle cell growth by the lipid peroxidation product 4-hydroxy-2-nonenal. *Circulation* **97**, 1071-1078.
27. Sastre, J., F. V. Pallardó, and J. Vina. 2000. Mitochondrial oxidative stress plays a key role in aging and apoptosis. *IUBMB Life* **49**, 427-435.
28. Satoh, K., Y. Ida, M. Tomioka, K. Shiohara, H. Sakagami, and S. Fujisawa. 1999. Interaction between antioxidants and hydroquinone/bisphenol. *Anticancer Res.* **19**, 5457-5462.
29. Shackelford, R. E., W. K. Kaufmann, and R. S. Paules. 2000. Oxidative stress and cell cycle checkpoint function. *Free Radic. Biol. Med.* **28**, 1387-1404.
30. Soh, Y., K. S. Jeong, I. J. Lee, M. A. Bae, Y. C. Kim, and B. J. Song. 2000. Selective activation of the c-Jun N-terminal protein kinase pathway during 4-hydroxynonenal-induced apoptosis of PC12 cells. *Mol. Pharmacol.* **58**, 535-541.
31. Tada, H., O. Shiho, K. Kuroshima, M. Koyama, and K. Tsukamoto. 1986. An improved colorimetric assay for interleukin 2. *J. Immunol. Methods* **93**, 157-165.
32. Uchida, K. 2003. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog. Lipid Res.* **42**, 318-343.
33. Walford, G. A., R. L. Moussignac, A. W. Scribner, J. Loscalzo, and J. A. Leopold. 2004. Hypoxia potentiates nitric oxide-mediated apoptosis in endothelial cells via peroxynitrite-induced activation of mitochondria-dependent and -independent pathways. *J. Biol. Chem.* **279**, 4425-4432.
34. Wyllie, A. H. 1995. The genetic regulation of apoptosis. *Curr. Opin. Genet. Dev.* **5**, 97-104.
35. Yoritaka, A., N. Hattori, K. Uchida, M. Tanaka, E. R. Stadtman, and Y. Mizuno. 1996. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc. Natl. Acad. Sci. USA* **93**, 2696-2701.

초록 : 4-Hydroxynonenal에 생성된 ROS와 peroxynitrite를 통한 내피세포의 세포사에 관한 연구

정상운 · 이수복 · 이지영 · 호세인 모하메드 악바르 · 김동환 · 장정윤 · 윤정현 · 정해영 · 김남득*
(부산대학교 약학대학 약학과)

지질과산화로부터 생성된 aldehyde 중 4-hydroxynonenal (HNE)는 산화적 손상과 관련된 다량의 arachidonic acids, linoleic acids 등으로부터 생성될 수 있다. 그러므로 HNE는 산화적 스트레스와 관련된 세포사에서 중요한 매개인자로 작용을 할 수가 있을 것이다. 본 연구는 HNE가 세포사를 유발할 것이라 가정하고, 먼저 흰쥐 전립선 유래 내피세포인 YPEN-1 세포에서 세포독성을 측정하였다. 세포생장 저해능력은 HNE를 5~15 μM 농도로 처리하여 형태적 변화와 MTT assay를 통하여 결과를 관찰하였다. 그 결과 HNE가 이 세포에서 핵형의 변화와 세포사를 유발시키는 것을 각각의 실험을 통해 확인이 되었다. 또한 이 사실을 단백질의 변화를 통하여 확인을 할 수가 있었다. HNE를 24시간 처리한 세포에서 poly(ADP-ribose) polymerase 단백질 분절이 매개되었고 Bax의 발현량이 증가하였다. 또한 세포내의 활성 산소종들을 발생시켰다. 이에 생성된 활성 산소종과 peroxynitrite가 세포사와 관련이 있는가를 밝히기 위하여 이들의 포식자들인 N-acetylcysteine과 penicillamine을 본 연구에서 사용하였다. 이들 포식자들에 의해 HNE에 의해 유도되는 세포사가 억제가 되었기에 산화적 활성화가 HNE에 의해 유도된 세포사와 관련이 있음을 알 수 있었다. 이러한 결과들은 HNE가 내피세포에서 ROS와 peroxynitrite 생성을 통하여 세포사를 일으킨다는 사실을 뒷받침해 준다.