SPANDIDOS PUBLICATIONS

Age-related differential growth rate and response to 4-hydroxynonenal in mouse aortic smooth muscle cells

TAE-JIN LEE[1], JUNG-TAE LEE[1], SUNG-KWON MOON[2], CHEORL-HO KIM[3], JONG-WOOK PARK[1] and TAEG KYU KWON[1]

[1]Department of Immunology and Chronic Disease Research Center and Institute for Medical Science, School of Medicine, Keimyung University, 194 DongSan-dong Jung-gu, Daegu 700-712; [2]Department of Food and Biotechnology, Chungju National University, Chungju, Chungbuk 380-702; [3]Department of Biochemistry and Molecular Biology, Dongguk University COM, Kyungju City, Kyungbuk 780-714, South Korea

Received August 25, 2005; Accepted September 28, 2005

Abstract. 4-Hydroxynonenal (4-HNE) is a peroxidation product of ω-6-poly-unsaturated fatty acids and exerts growth modifying as well as cytotoxic activities. This aldehyde component of oxidized lipid is increased during the aging process. In this study, to characterize the potential role of the lipid peroxidation product in aging, we studied the effects of 4-HNE on cell proliferation and activation of cell-cycle machinery and the mitogen-activated protein kinase signaling pathway. 4-HNE-treated smooth muscle cells (SMCs) have shown a different cell proliferation rate depending on 4-HNE's incubation time and concentration. Interestingly, a prolonged treatment of 0.1 μM 4-HNE (36 h) resulted in an increase of cell growth in young SMCs but displayed cytotoxicity in aged SMCs. Treatment with 4-HNE enhanced cyclin D1 expression and activation of the extracellular signal-regulated kinase (ERK) signaling pathway, which were stronger in young SMCs compared with aged SMCs. Moreover, 4-HNE-induced cell proliferation and cyclin D1 expression were significantly attenuated by PD98059, the ERK inhibitor, in young SMCs. These data clearly indicate that increased cell proliferation was associated with the induction of cyclin D1 expression which was regulated by ERK in 4-HNE-treated young SMCs for 36 h. In contrast, we found that the cytotoxicity of aged SMCs to 4-HNE was partly related to generation of ROS and that pretreatment with N-acetyl-L-cysteine prevented 4-HNE-induced cell death in aged SMCs. These results suggest that the prolonged treatment of 0.1 μM 4-HNE-induced cell growth inhibition was caused by generation of ROS. Collectively, the age-related different growth rates and responses to 4-HNE are related to the expression level of cyclin D1, activation of the ERK signaling pathway, and regulation of ROS generation in SMCs.

Correspondence to: Dr Taeg Kyu Kwon, Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-dong Jung-gu, Daegu 700-712, South Korea
E-mail: kwontk@kmu.ac.kr

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; 4-HNE, 4-hydroxynonenal; MAPKs, mitogen-activated protein kinases; SMCs, smooth muscle cells

Key words: cyclin D1, ERK, 4-hydroxynonenal, SMCs

Introduction

Aging is an inevitable biological process which is characterized by a general decline in physiological function. During the aging process, free radical damages accumulate and anti-oxidant capacities change, which is associated with oxidative stress in cells and tissue (1). The accumulation of free radical damage and changes in antioxidant capacity appear to induce degenerative disease, atherosclerosis, neurodegenerative disease, ischemia-reperfusion and cancer (2,3). One of the major targets of oxidative damage is cellular membrane lipid (4). During the process of lipid peroxidation, it is known to produce many reactive species that are associated with oxidative stress in cells and tissue (5). Lipid peroxidation is associated with the pathogenesis of numerous diseases, including atherosclerosis, diabetes, cancer, and rheumatoid arthritis, as well as with aging (2,3,6).

4-HNE is a major lipid peroxidation product of n-6-ployunsaturated fatty acids, such as arachidonic acid and linoleic acid. Among several reactive lipid aldehydes, 4-HNE is involved in various biological processes associated with oxidative stress, modulation of cell signaling pathways and the induction of apoptosis (7,8). 4-HNE is generated in relatively large amounts at concentrations of 10 μM to 5 mM in response to oxidative insults, which is believed to be largely responsible for the cytopathological effects observed during oxidative stress *in vivo* (5). 4-HNE exhibits a wide range of biological activities that affect signaling cascades in a concentration-dependent manner. A moderately high concentration of 4-HNE can induce apoptosis (4,9) and differentiation (10). On the other hand, low levels of 4-HNE promote cell proliferation (11,12). Previous reports have shown that 4-HNE stimulated rat (12) and mouse aortic SMC growth (13).

In the present study, we hypothesize that 4-HNE could induce different effects on cell proliferation, cell death, and the MAPK cell signaling cascade affected by aging in SMCs. To characterize the mechanisms of differential modulation of 4-HNE-induced mitogenesis and cell death, we analyzed its effects on the expression of cell-cycle machinery, activation of the MAPK signaling pathway, and ROS generation in young and aged SMCs.

Materials and methods

Cell culture. Mouse SMCs were isolated from young (4 months old) and aged (16 months old) mouse aortas by enzyme digestion, as previously described in detail (13). In this study, cells were grown in DMEM containing 10% heat-inactivated FBS. Cells were used within five passages of primary culture in experiments.

Proliferative capacities assay. $1x10^6$ young and aged SMCs were inoculated in 100-mm plates in complete DMEM culture media. Cells were trypsinized at the indicated time and the cell number was determined using the trypan blue exclusion method. Three independent experiments were performed for each experimental condition.

Cell proliferation assay. Cells were initially grown in 100-mm plates in complete culture media for 24 h and, after washing with PBS, were incubated in serum-free DMEM media for 48 h. Cells were treated with 0.1 μM 4-HNE (Cayman Chemical, Ann Arbor, MI) in the presence of 0.1% FBS for 12, 24, and 36 h. At the end of the incubation periods, cells were trypsinized and the cell number was determined using the trypan blue exclusion method.

Cell counting kit-8 (CCK-8) assay. CCK-8 assay is nearly proportional to the cell number and, thus, can be used to determine proliferation and cytotoxicity. Cells were plated at a concentration of 10,000 cells in 0.1 ml of 10% FBS-DMEM per well in 96-well plates. After 24 h incubation, cells were serum-starved for 48 h and treated with media containing 0.1% FBS alone or supplemented with 4-HNE (0.1-10 μM), or a combination of 4-HNE with the inhibitor (5 μM PD98059). The cells were then grown for an additional 36 h, and the CCK-8 assay was carried out using the manufacturer's instructions.

Western blotting. 4-HNE-treated cells were harvested using a cell scraper in the presence of PBS and lysed in 0.1 ml of lysis buffer (20 mM PIPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, pH 7.4) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovandate). The collected lysate in Eppendorf tubes was placed on ice for 30 min. After centrifugation (20 min; 16,000 g at 4˚C), supernatants were removed and the protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). Protein from each sample (50 μg) was resolved on 12% Tris-glycine gels under reducing conditions, and transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in TBS-T (150 mM NaCl, 200 mM Tris, 0.1% Tween-20) for 1 h and then incubated with one of the following primary antibodies: cyclin D1, cyclin A, CDK4, actin, phospho-ERK, -JNK, p38-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation at room temperature, membranes were washed with TBS-T and incubated with a 1:1000 dilution of HRP-conjugated secondary antibody (1:1000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Membranes were then washed for 30 min, treated with the ECL detection system (Amersham, Arlington Heights, IL), and exposed X-ray film.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from SMCs using the TRI reagent (Molecular Research Center, Cincinnati, OH). cDNA was synthesized from 2 μg total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The 50 μl PCR reaction mixture contained 36.6 μl H_2O, 5 μl 10X PCR buffer, 3 μl 25 mM $MgCl_2$, 0.4 μl 100 mM dNTP mix, 1 μl both sense and antisense primers, and 0.5 μl *Taq* polymerase (5 units/μl). The primers for cyclin D1 and actin were synthesized by Genomine Inc. (South Korea). The sequence of the primers was as follows: cyclin D1 sense, 5'-AGTGCGTGCAGAAGGAGATT-3'; cyclin D1 antisense, 5'-CACAACTTCTCGGCAGTCAA-3'; actin sense, 5'-ACG ACATGGAGAAGATCTGGCACC-3'; and actin antisense, 5'-TCAGGCAGCTCATAGCTCTTCTC-3'. PCR was carried out under the following conditions: 20 cycles of 95˚C for 1 min, 58˚C for 1.5 min, and 72˚C for 1.5 min with a final 10 min extension cycle at 72˚C.

Measurement of reactive oxygen species (ROS) generation. The generation of ROS was measured by flow cytometry analysis using H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) as a substrate. Briefly, SMCs were incubated with 0.1 μM 4-HNE for 36 h, loaded with 10 μM H_2DCFDA 1 h prior to harvesting. The fluorescence was measured at the desired time intervals by flow cytometry. ROS generation was assessed by the DCF fluorescence intensity (FL-1, 530 nm) from 10,000 cells using a FACS Caliber flow cytometer (Becton-Dickinson).

Statistical analysis. Quantitative results are given as means ± SE. For statistical evaluation, the results were analyzed using the Student's t-test. Values of $P<0.05$ were considered statistically significant.

Results

Modulation of 4-HNE on young and aged SMC proliferation. To compare the proliferative capacities of young and aged SMCs, cells were cultured in complete media containing 10% FBS for 72 h. Cells were harvested and counted using the trypan blue exclusion method after 24, 48, and 72 h. As shown in Fig. 1A, the cell number of young SMCs increased up to 2.5-fold compared with that of aged SMCs after 72 h. This result indicates that young SMCs showed a higher growth rate compared with that of aged SMCs.

To evaluate the effects of 4-HNE on young and aged SMC proliferation, we analyzed the total cell number using the trypan blue exclusion method. After exposure of growth-

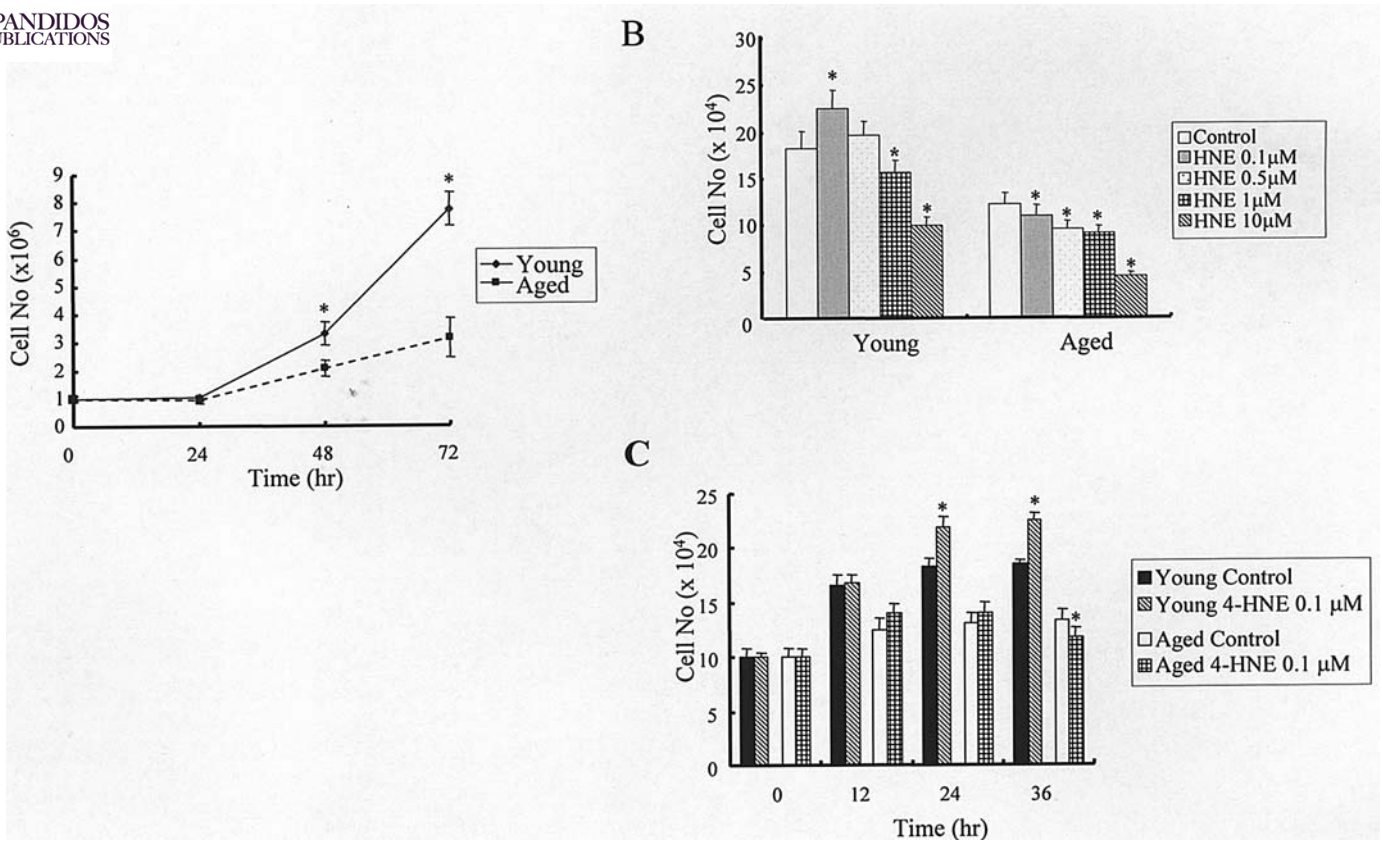


Figure 1. Effect of 4-HNE on young and aged SMC proliferation. (A) Proliferative capacity of SMCs. Young and aged SMCs were plated at equal densities overnight and cultured in standard growth media for 72 h. Cells were counted in triplicate for the indicated periods of time. Results are presented as mean ± SE for three independent experiments. *P<0.05 compared with aged SMCs. (B) Dose effect of 4-HNE on SMC proliferation. Growth-arrested SMCs were treated with various concentrations of 4-HNE for 36 h and cells were counted in triplicate for the indicated periods of time. Results are presented as mean ± SE for three independent experiments. *P<0.05 compared with control. (C) Time effect of 4-HNE on SMC proliferation. Growth-arrested SMCs were treated with 0.1 μM 4-HNE for the indicated times. Cells were counted in triplicate for the indicated periods of time. Results are presented as mean ± SE for three independent experiments. *P<0.05 compared with control.

arrested young and aged SMCs to 4-HNE (0.1-10 μM) for 36 h, the cell number was determined. The cell number was increased after treatment with 0.1 μM or 0.5 μM 4-HNE (27% and 15%, respectively, P<0.05) compared with controls in young SMCs (Fig. 1B). In contrast, the cell number of aged SMCs decreased in a dose-dependent manner (Fig. 1B). In addition, we analyzed the time kinetic effect of 0.1 μM 4-HNE on young and aged SMC proliferation using the trypan blue exclusion method after treatment for 12, 24, and 36 h. As shown in Fig. 1C, treatment with 0.1 μM 4-HNE increased the cell proliferation in young and aged SMCs by 24 h, but decreased it in aged SMCs after 36 h. This result indicates that short exposure to 4-HNE induces cell proliferation but prolonged exposure inhibits cell growth in aged SMCs and 4-HNE induces different effects on cell proliferation or cell death depending on age.

Effect of 4-HNE on the expression of cell-cycle related proteins in young and aged SMCs. To determine whether age-dependent cell proliferation in 4-HNE-treated SMCs is related to the expression of cell-cycle machinery, protein extracts were prepared from cells treated for indicated time points. We found that 4-HNE treatment caused an increase in cyclin D1 protein levels without detectable changes in cyclin A and CDK4 protein levels in young SMCs (Fig. 2A). However, cyclin D1 expression was slightly increased by 24 h but decreased after 36 h in 4-HNE-treated aged SMCs (Fig. 2A). To investigate the mechanism by which 4-HNE treatment increased cyclin D protein levels, we measured the effect of 4-HNE on steady state cyclin D1 mRNA levels. Total RNA was isolated from SMCs after treatment with 0.1 μM 4-HNE for 12, 24 and 36 h. The levels of mRNA transcript of cyclin D1 were analyzed by RT-PCR. While the expression levels of cyclin D1 mRNA had peaked at 12 h and were sustained until 36 h in young SMCs, the expression level of cyclin D1 slightly increased in aged SMCs by 24 h but decreased after 36 h (Fig. 2B). These data clearly indicate that induction of cyclin D1 expression was regulated by 4-HNE in a transcriptional level.

4-HNE-induced activation of ERK is stronger in young SMCs than in aged SMCs. To investigate the mechanisms involved in stimulation of growth in SMCs induced by 0.1 μM 4-HNE, we decided to evaluate the activation of MAPK signaling pathways including ERK in 4-HNE-treated-SMCs. The phosphorylation levels of ERK, JNK, and p38 were detected by Western blot analysis using their phospho-specific antibodies. Only 4-HNE-induced ERK activation was significantly increased in young and aged SMCs, which was maximally activated after treatment for 90 min (Fig. 3A). The activation

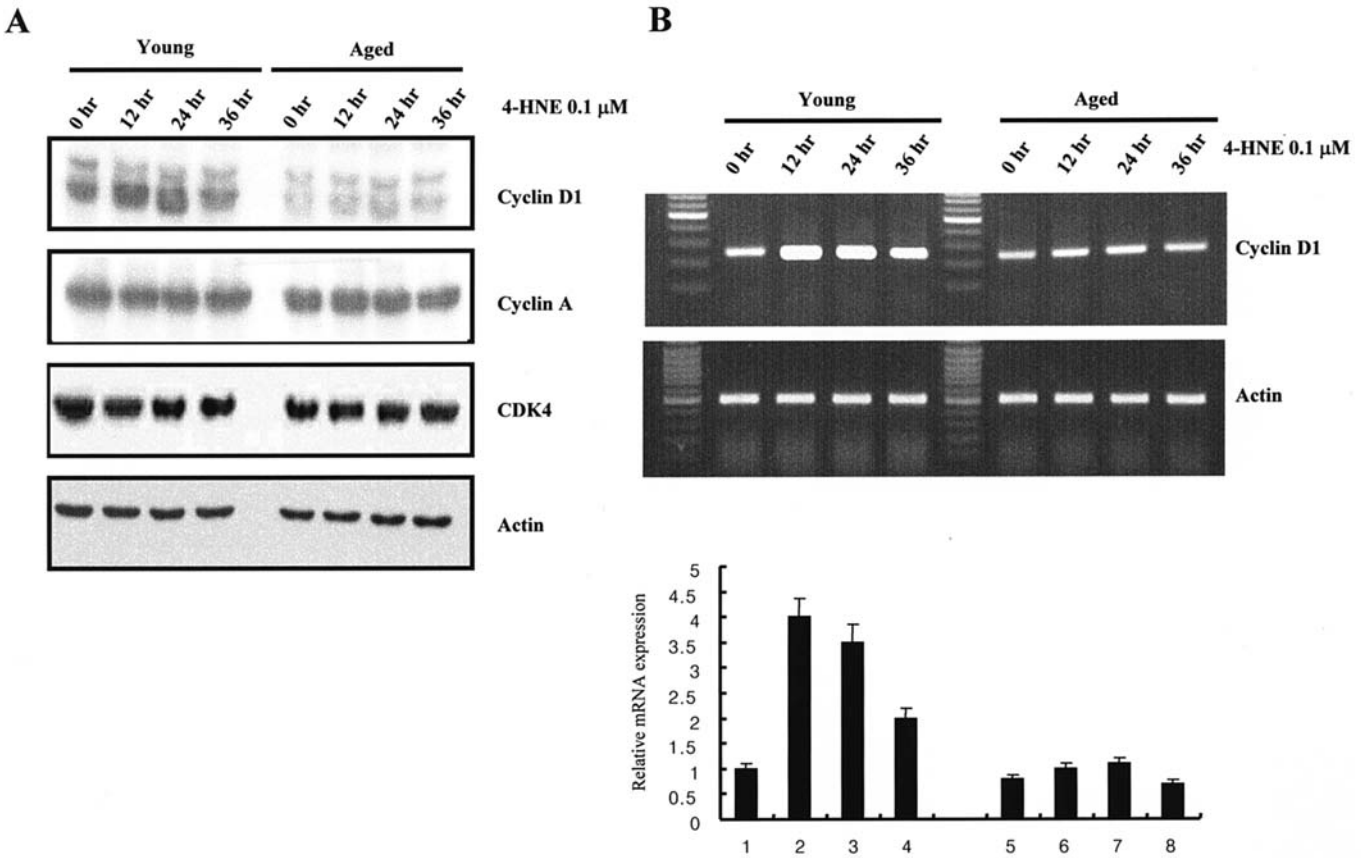
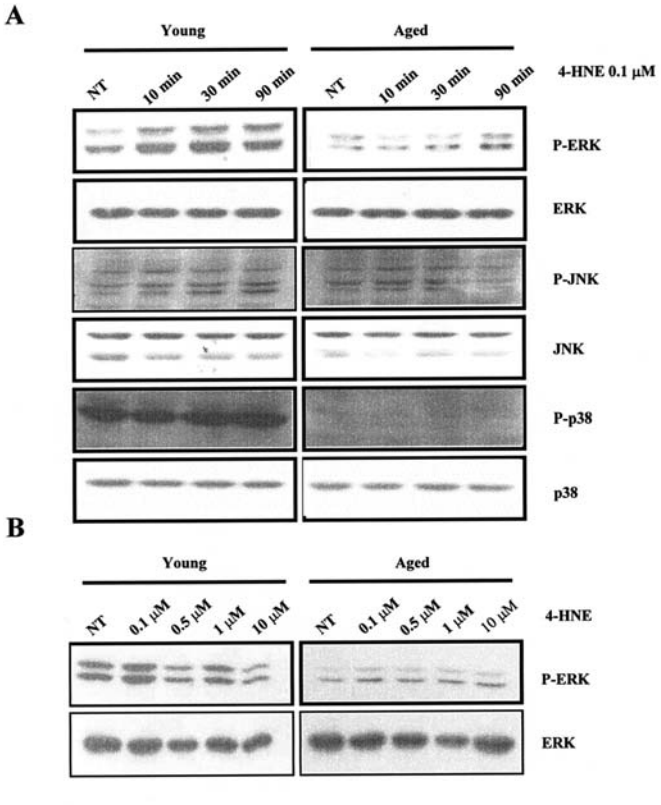


Figure 2. Effects of 4-HNE on the expression levels of cell-cycle regulatory proteins in SMCs. (A) Cell lysates were prepared as described in Materials and methods. Equal amounts of whole cell lysate (50 μg) were subjected to electrophoresis and analyzed by Western blotting for cyclin D1, cyclin A, CDK4 and actin. (B) Effect of 4-HNE on mRNA expression of cyclin D1. SMCs were treated with 0.1 μM 4-HNE for the indicated periods of time. Total cellular RNA was extracted from 4-HNE-treated SMCs and RT-PCR analysis was performed using primers specific for cyclin D1 and actin. A representative result is shown; two additional experiments yielded similar results. The bottom panel showed cyclin D1 mRNA levels normalized to actin mRNA levels.



intensities of phospho-ERK were higher in young SMCs than in aged SMCs (Fig. 3A). However, 4-HNE treatment slightly increased the phosphorylation status of JNK but not p38 MAPK (Fig. 3A). In dose-response experiments, young and aged SMCs were treated with various concentrations of 4-HNE for 90 min as indicated in Fig. 3B. The increase in ERK phosphorylation peaked at 0.1 μM 4-HNE and decreased at a high dose of 4-HNE (above 0.5 μM). The intensity of ERK activation by 4-HNE was higher in young SMCs than in aged SMCs (Fig. 3B).

The mitogenic effect of 4-HNE is at least partly mediated by the ERK signaling pathway in young SMCs. We focused on the role of 4-HNE affected by aging in increasing cell proliferation

Figure 3. Effects of 4-HNE on phosphorylation of MAPKs in SMCs. (A) Time effect of 4-HNE on phosphorylation of MAPKs. SMCs were stimulated with 0.1 μM 4-HNE for the indicated periods of time. The whole-cell lysates were analyzed by immunoblot analysis using various antibodies against the phospho-MAPKs. To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies against the corresponding total MAPK. (B) Dose effects of 4-HNE on phosphorylation of ERK. SMCs were stimulated with the indicated concentrations of 4-HNE for 90 min. The whole-cell lysates were analyzed by immunoblot analysis using various antibodies against the phospho-ERK.

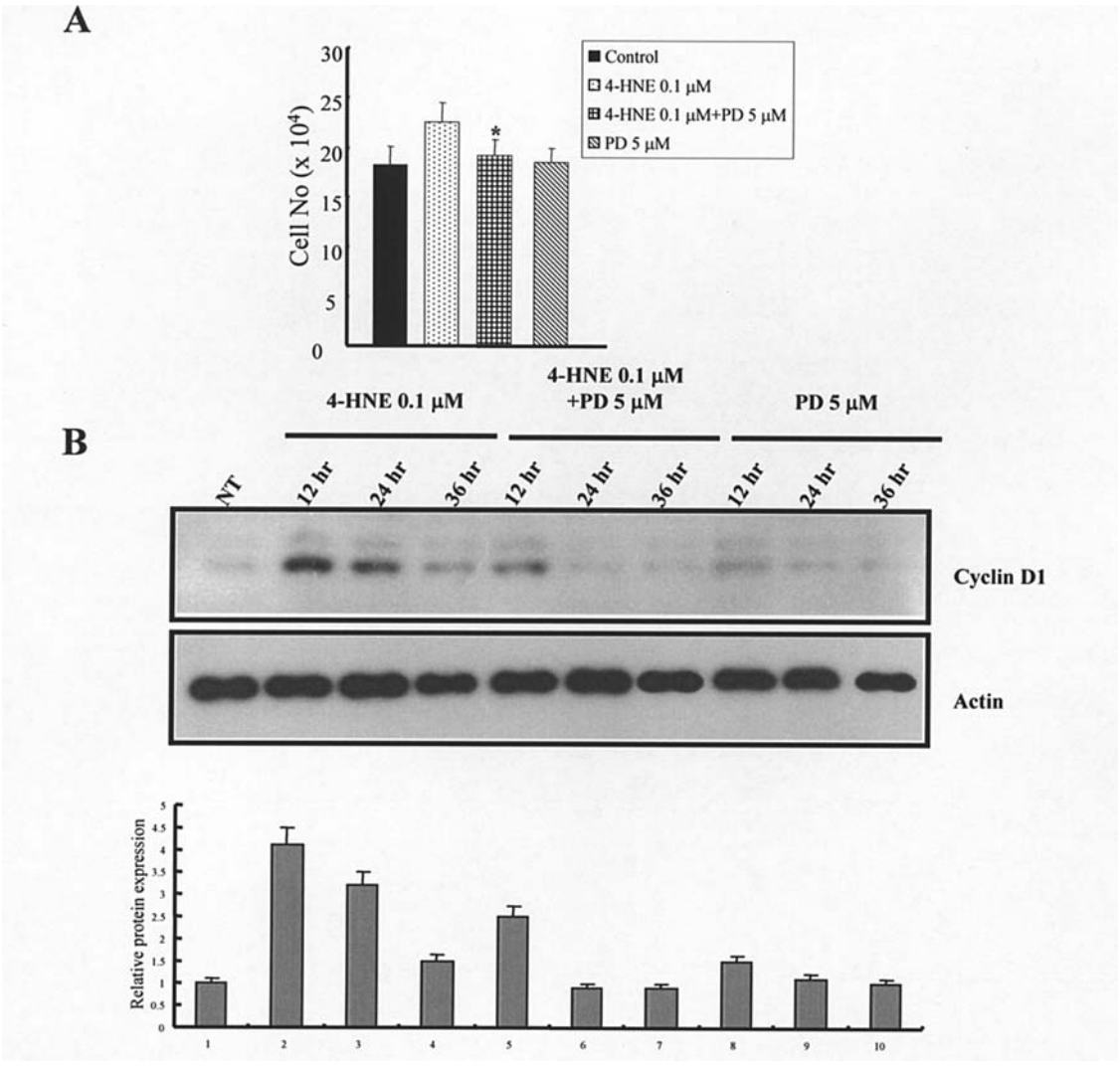


Figure 4. Effects of ERK inhibitor on cell proliferation and cyclin D1 expression in young SMCs. (A) 4-HNE-induced cell proliferation was inhibited by PD98059 in young SMCs. Five μM PD98059 pretreated-SMCs were stimulated with 0.1 μM 4-HNE for 36 h and cells were counted in triplicate for 36 h. Results are presented as mean ± SE for three independent experiments. *P<0.05 compared with 4-HNE-treated SMCs. (B) 4-HNE-induced increase of cyclin D1 expression was reduced by PD98059. The whole-cell lysates were analyzed by immunoblot analysis using cyclin D1 antibody. For normalization of loading, blots were stripped and reprobed with the antibody against the corresponding actin. The bottom panel shows cyclin D1 protein levels normalized to actin protein levels.

or inhibiting cell growth in SMCs. To study the mechanism of 4-HNE to induce cell proliferation in young SMCs, we examined the action of the specific inhibitor, PD98059, on young SMC proliferation and expression of cyclin D1. Pretreatment of 5 μM PD98059 abrogated 4-HNE-induced cell proliferation in young SMCs that was not inhibited by PD98059 single treatment (Fig. 4A). To confirm the relationship between the ERK signaling pathway and cyclin D1 expression in 4-HNE-treated SMCs, cells were pretreated with PD98059 and incubated in the presence of 0.1 μM 4-HNE for 36 h. As shown in Fig. 4B, pretreatment of 5 μM PD98059 attenuated 4-HNE-induced cyclin D1 expression in young SMCs, suggesting that the mitogenic effect of 4-HNE is regulated by the ERK signaling pathway.

4-HNE-induced cell death is mediated through the generation of ROS in aged SMC. To verify that observed decreases in aged SMC viability in response to 4-HNE treatment were due to generation of ROS, we analyzed the production of ROS in 4-HNE-treated SMCs (Fig. 5A). During the aging of many cell types, there appears to be an increased level of ROS generation (14-16). After 4-HNE treatment, H_2O_2 levels were increased in aged SMCs but not young SMCs (Fig. 5B). We then examined the relationship between ROS generation and cell death in 4-HNE-treated aged SMCs. Pretreatment with NAC prevented cell death induced by 4-HNE in aged SMCs, suggesting that 4-HNE-induced cell death was related to generation of ROS in aged SMCs (Fig. 5C).

Discussion

The present study was initiated to determine whether 4-HNE may increase cell proliferation in mouse SMCs and to compare the ability of 4-HNE-induced cell proliferation and cell death between young and aged SMCs. Consistent with previous observations, we found that 4-HNE was toxic to cells at high concentrations while lower concentrations induced cell growth (Fig. 1B). Interestingly, 0.1 μM 4-HNE induced a reverse

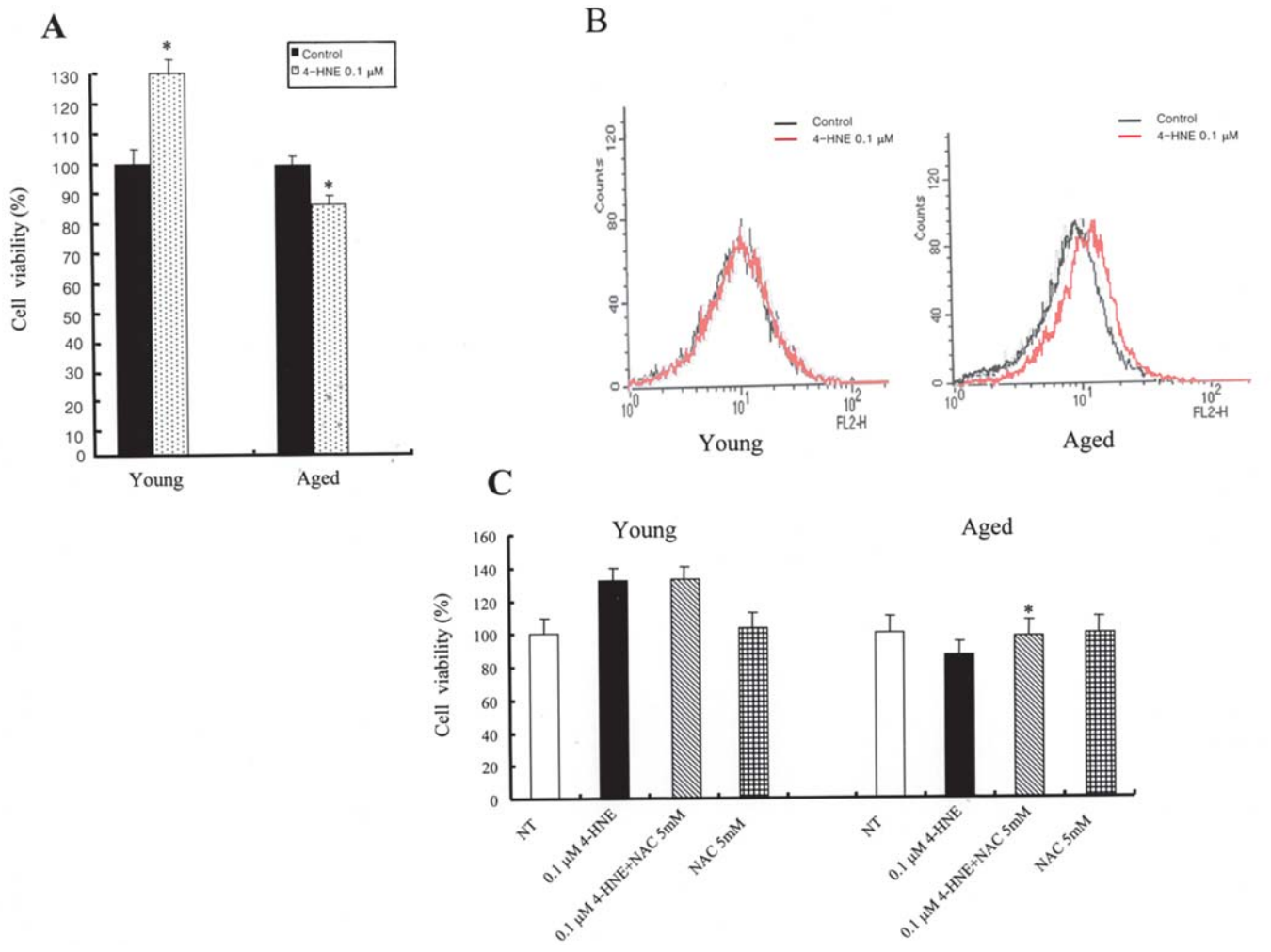


Figure 5. Effects of ROS scavenger, NAC, on 4-HNE-induced cell death in aged SMCs. (A) Treatment with 4-HNE induced cell death in aged SMCs. Growth-arrested SMCs were treated with 0.1 μM 4-HNE for 36 h and the cell number was analyzed using a CCK-8 kit. Results are presented as mean ± SE for three independent experiments. *P<0.05 compared with control. (B) Treatment with 4-HNE provoked production of ROS in aged SMCs but not in young SMCs. SMCs were incubated with 0.1 μM 4-HNE for 36 h, loaded with 10 μM H_2DCFDA 1 h prior to harvesting. The fluorescence was measured at the desired time intervals by flow cytometry. (C) 4-HNE-induced cell death is mediated through the generation of ROS in aged SMCs. SMCs were pretreated for 1 h with the indicated doses of NAC, treated with 4-HNE for an additional 36 h, and analyzed using CCK-8 assay. Values are expressed as percentage of control, and each bar represents the mean ± SE of triplicate determinations. *P<0.05 compared with 4-HNE-treated aged SMCs.

effect in prolonged 4-HNE-treated SMCs which increased cell proliferation in young SMCs but inhibited cell growth in aged SMCs (Fig. 1C). This result suggests that 4-HNE could elicit opposite responses based on its exposure time and aging in SMCs.

To assure the differences in response to 4-HNE in young and aged SMCs, we examined the expression level of cell-cycle related protein, activation of MAPK, and ROS generation in 4-HNE-treated SMCs. Firstly, to elucidate that the specific cell-cycle regulatory proteins are responsible for an age-dependent differential cell proliferation rate and response by 4-HNE, we examined the expression levels of cyclin D1, cyclin A, and CDK4 proteins. As shown in Fig. 2A, cyclin D1 expression was elevated in young SMCs without any change in cyclin A and CDK4 proteins. In contrast, cyclin D1 expression was slightly increased by 24 h but decreased after 36 h in 4-HNE-treated aged SMCs (Fig. 2A). Also, we have found that cyclin D1 mRNA was induced in 4-HNE-treated SMCs, suggesting that elevation of cyclin D1 protein may be regulated in transcriptional level (Fig. 2B). Moon *et al* reported that the responsiveness to 4-HNE or α-thrombin was greater in young SMCs compared with aged SMCs, which was caused by differential expression of cyclin D1 (13). Together, differential cell proliferation rate and response to 4-HNE were partly related to levels of cyclin D1 expression in SMCs.

The MAPK signaling cascade has been shown to regulate a wide variety of cellular events such as cell proliferation, differentiation and development. In previous studies, it was reported that 4-HNE induced rat SMC and T-cell growth through activation of ERK, JNK, p38 MAPK pathways (12,17-19). In our study, 4-HNE activated the ERK signal pathway and slightly increased the phosphorylation status of JNK but not p38 pathways (Fig. 3A). The occurrence of MAPK activation in discrepancy may be explained by differences of 4-HNE concentration. The intensity of ERK activation was stronger in young SMCs than in aged SMCs.

It was reported that cyclin D1 expression is associated with activation of ERK following platelet-derived growth factor stimulation and that cyclin D1, which is a key regulator of G1

on in mammalian cells, is also a key downstream ERKs (20). In the present study, we examined whether the differential activation of ERK was important for the regulation of cell growth and cyclin D1 expression in SMCs. We observed that 4-HNE treatment activates the ERK signal pathway and that the inhibition of this signaling pathway, using a specific ERK inhibitor (PD98059), significantly reduced 4-HNE-induced SMC proliferation and cyclin D1 expression in young SMCs (Fig. 4). In aged SMCs, treatment with PD98059 decreased the expression of cyclin D1 and potentiated 4-HNE-induced cell death (data not shown). These results show that 4-HNE stimulates SMC proliferation requiring activation of ERK and that cyclin D1 is a key downstream target of ERKs in 4-HNE-treated SMCs. Together with our data, previous studies have shown that cell proliferation and cyclin D1 gene activation is dependent upon the activation of MAPK (21,22). Further investigation is needed to provide direct evidence of the ability of ERK to increase cell proliferation in 4-HNE-treated SMCs.

Finally, to elucidate the mechanism of cell-growth inhibition in 0.1 μM 4-HNE-treated aged SMCs, we analyzed the relationship between ROS production and cell death. As shown in Fig. 5B and C, ROS generation was increased in aged SMCs but not in young SMCs and pretreatment of aged SMCs with NAC inhibited 4-HNE-induced cell death, indicating that 4-HNE-induced cell death was mediated by ROS generation. To elucidate whether 4-HNE-induced cell death was dependent on the caspase pathway, we analyzed caspase-3 activity. We did not detect any changes in caspase-3 activity in 4-HNE-treated SMCs (data not shown). Therefore, 4-HNE-induced growth inhibition in prolonged 4-HNE-treated aged SMCs could be caused by cell death that is mediated by the caspase-3 independent pathway through a generation of ROS. Additional experiments are necessary to determine the possible mechanism of cell death in prolonged 4-HNE-treated aged SMCs. Our future aim is to define the biological consequences of age-related different responses to 4-HNE that could be linked to age-related various degenerative processes, including vascular dysfunction.

In conclusion, we have shown that 4-HNE exerts differential modulation on cell proliferation that is mediated, at least in part, through cyclin D1 and the ERK signaling pathway in young SMCs and induces cell death through a generation of ROS in aged SMCs.

Acknowledgements

This study was supported by a grant from Korea Research Foundation (KRF-2002-015-DS0002).

References

1. Lee JH, Jung KJ, Kim JW, Kim HJ, Yu BP and Chung HY: Suppression of apoptosis by calorie restriction in aged kidney. Exp Gerontol 39: 1361-1368, 2004.
2. Halliwell B: Tell me about free radicals, doctor: a review. J R Soc Med 82: 747-752, 1989.
3. Halliwell B: Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. Br J Exp Pathol 70: 737-757, 1989.
4. Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y and Osawa T: Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. J Biol Chem 274: 2234-2242, 1999.
5. Esterbauer H, Schaur RJ and Zollner H: Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11: 81-128, 1991.
6. Uchida K: 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res 42: 318-343, 2003.
7. Shackelford RE, Kaufmann WK and Paules RS: Oxidative stress and cell cycle checkpoint function. Free Radic Biol Med 28: 1387-1404, 2000.
8. Haynes RL, Brune B and Townsend AJ: Apoptosis in RAW 264.7 cells exposed to 4-hydroxy-2-nonenal: dependence on cytochrome *c* release but not p53 accumulation. Free Radic Biol Med 30: 884-894, 2001.
9. Yang Y, Sharma A, Sharma R, Patrick B, Singhal SS, Zimniak P, Awasthi S and Awasthi YC: Cells preconditioned with mild, transient UVA irradiation acquire resistance to oxidative stress and UVA-induced apoptosis: role of 4-hydroxynonenal in UVA-mediated signaling for apoptosis. J Biol Chem 278: 41380-41388, 2003.
10. Barrera G, Di Mauro C, Muraca R, Ferrero D, Cavalli G, Fazio VM, Paradisi L and Dianzani MU: Induction of differentiation in human HL-60 cells by 4-hydroxynonenal, a product of lipid peroxidation. Exp Cell Res 197: 148-152, 1991.
11. Kreuzer T, Grube R, Wutte A, Zarkovic N and Schaur RJ: 4-Hydroxynonenal modifies the effects of serum growth factors on the expression of the c-fos proto-oncogene and the proliferation of HeLa carcinoma cells. Free Radic Biol Med 25: 42-49, 1998.
12. Ruef J, Rao GN, Li F, Bode C, Patterson C, Bhatnagar A and Runge MS: Induction of rat aortic smooth muscle cell growth by the lipid peroxidation product 4-hydroxy-2-nonenal. Circulation 97: 1071-1078, 1998.
13. Moon SK, Thompson LJ, Madamanchi N, Ballinger S, Papaconstantinou J, Horaist C, Runge MS and Patterson C: Aging, oxidative responses, and proliferative capacity in cultured mouse aortic smooth muscle cells. Am J Physiol Heart Circ Physiol 280: H2779-H2788, 2001.
14. Fischer BM and Voynow JA: Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. Am J Respir Cell Mol Biol 26: 447-452, 2002.
15. Shao MX and Nadel JA: Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. Proc Natl Acad Sci USA 102: 767-772, 2005.
16. Aoshiba K, Yasuda K, Yasui S, Tamaoki J and Nagai A: Serine proteases increase oxidative stress in lung cells. Am J Physiol Lung Cell Mol Physiol 281: L556-L564, 2001.
17. Kakishita H and Hattori Y: Vascular smooth muscle cell activation and growth by 4-hydroxynonenal. Life Sci 69: 689-697, 2001.
18. Whisler RL, Newhouse YG and Bagenstose SE: Age-related reductions in the activation of mitogen-activated protein kinases p44mapk/ERK1 and p42mapk/ERK2 in human T cells stimulated via ligation of the T cell receptor complex. Cell Immunol 168: 201-210, 1996.
19. Gorgas G, Butch ER, Guan KL and Miller RA: Diminished activation of the MAP kinase pathway in CD3-stimulated T lymphocytes from old mice. Mech Ageing Dev 94: 71-83, 1997.
20. Ramakrishnan M, Musa NL, Li J, Liu PT, Pestell RG and Hershenson MB: Catalytic activation of extracellular signal-regulated kinases induces cyclin D1 expression in primary tracheal myocytes. Am J Respir Cell Mol Biol 18: 736-740, 1998.
21. Bhatt KV, Spofford LS, Aram G, McMullen M, Pumiglia K and Aplin AE: Adhesion control of cyclin D1 and p27Kip1 levels is deregulated in melanoma cells through BRAF-MEK-ERK signaling. Oncogene 24: 3459-3471, 2005.
22. Shukla N, Rowe D, Hinton J, Angelini GD and Jeremy JY: Calcium and the replication of human vascular smooth muscle cells: studies on the activation and translocation of extracellular signal regulated kinase (ERK) and cyclin D1 expression. Eur J Pharmacol 509: 21-30, 2005.