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Low concentration of 4-hydroxy hexenal increases heme oxygenase-1 expression through activation of Nrf2 and antioxidative activity in vascular endothelial cells

Atsushi Ishikado [a], Yoshihiko Nishio [a,*], Katsutaro Morino [a], Satoshi Ugi [a], Hajime Kondo [a], Taketoshi Makino [b], Atsunori Kashiwagi [a], Hiroshi Maegawa [a]

[a] Division of Endocrinology and Metabolism, Department of Medicine, Shiga University of Medical Science, Seta-Tsukinowa-Cho, Otsu, Shiga 520-2192, Japan
[b] Research & Development Department, Sunstar Inc., 3-1, Asahi-Machi, Takatsuki, Osaka 569-1195, Japan

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ABSTRACT

Large-scale clinical studies have shown that *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) such as eicosapentaenoic and docosahexaenoic acids reduce cardiovascular events without improving classical risk factors for atherosclerosis. Recent studies have proposed that direct actions of *n*-3 PUFAs themselves, or of their enzymatic metabolites, have antioxidative and anti-inflammatory effects on vascular cells. Although a recent study showed that plasma 4-hydroxy hexenal (4-HHE), a peroxidation product of *n*-3 PUFA, increased after supplementation of docosahexaenoic acid, the antiatherogenic effects of 4-HHE in vascular cells remain unclear.

In the present study, we tested the hypothesis that 4-HHE induces the antioxidative enzyme heme oxygenase-1 (HO-1) through activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulatory transcriptional factor, and prevents oxidative stress-induced cytotoxicity in vascular endothelial cells. This mechanism could partly explain the cardioprotective effects of *n*-3 PUFAs.

Human umbilical vein endothelial cells were stimulated with 1–10 μM 4-HHE or 4-hydroxy nonenal (4-HNE), a peroxidation product of *n*-6 PUFAs. Both 4-HHE and 4-HNE dose-dependently increased HO-1 mRNA and protein expression, and intranuclear expression and DNA binding of Nrf2 at 5 μM. Small interfering RNA for Nrf2 significantly reduced 4-HHE- or 4-HNE-induced HO-1 mRNA and protein expression. Furthermore, pretreatment with 4-HHE or 4-HNE prevented *tert*-butyl hydroperoxide-induced cytotoxicity.

In conclusion, 4-HHE, a peroxidation product of *n*-3 PUFAs, stimulated expression of the antioxidant enzyme HO-1 through the activation of Nrf2 in vascular endothelial cells. This resulted in prevention of oxidative stress-induced cytotoxicity, and may represent a possible mechanism to partly explain the cardioprotective effects of *n*-3 PUFAs.

1. Introduction

Long-chain *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), such as eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), contained in fish oil were shown to reduce cardiovascular disease in epidemiological studies of Eskimos in the 1970s [1,2]. Recent large-scale interventional and cross-sectional studies have shown that *n*-3 PUFAs reduce cardiovascular events independently of the classical risk factors for atherosclerosis [3–5], suggesting direct antioxidative or anti-inflammatory effects of *n*-3 PUFAs on vascular tissue. Many studies have already shown that *n*-3 PUFAs themselves demonstrate a variety of bioactivities such as anti-inflammatory [6,7] and antioxidative effects [8], improvement of endothelial function [9], and suppression of monocyte adhesion in vascular tissue [7,10], explaining their antiatherogenic effects. In a recent study, enzymatic lipid metabolites of *n*-3 PUFAs, including resolvin and protectin, were shown to exert potent anti-inflammatory effects in aortic endothelial cells, leading to atheroprotection [11]. Furthermore, Calzada et al. demonstrated that plasma levels of 4-hydroxy hexenal (4-HHE), as an end-product aldehyde of *n*-3 PUFA peroxidation, were elevated following supplementation with 800 or 1600 mg/day DHA, although this level comprised 0.01% of the DHA present in the plasma [12]. However, the role of 4-HHE in vascular tissue remains incompletely understood.

Abbreviations: *n*-3 PUFA, *n*-3 polyunsaturated fatty acid; 4-HHE, 4-hydroxy hexenal; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; 4-HNE, 4-hydroxy nonenal; Keap1, Kelch-like ECH-associated protein 1; NQO1, NAD(P)H quinone oxidoreductase 1; ARE, antioxidant response element; tBHP, *tert*-butyl hydroperoxide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

* Corresponding author. Fax: +81 77 543 3858.
E-mail address: nishio@belle.shiga-med.ac.jp (Y. Nishio).

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Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive master regulatory transcriptional factor that plays an important atheroprotective role in vascular endothelial cells by regulating endothelial redox balance [13–15]. In unstimulated cells, Nrf2 resides in the cytoplasm bound to Kelch-like ECH-associated protein 1 (Keap1). Shear stress, dietary antioxidants, or other physiological stimuli that disrupt Keap1-Nrf2 interactions cause nuclear translocation of Nrf2, resulting in the transcription of antioxidant and phase II defense enzymes such as gammaglutamyl-cysteine ligase, NAD(P)H quinone oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1), through binding to the antioxidant response element (ARE) consensus sequence [16,17]. HO-1, a rate-limiting enzyme in heme metabolism, has been recognized as an important factor protecting vascular tissue against atherosclerosis by exerting antioxidative, anti-inflammatory, antiproliferative, anti-apoptotic and vasodilatory effects on the vasculature [18]. HO-1 converts heme into vasculoprotective carbon monoxide (CO) and biliverdin. Biliverdin, a potent antioxidant, is also known to prevent oxidative stress-induced atherogenesis [19]. Several studies in animal models have also emphasized the importance of HO-1 activity as a potent cardioprotective factor in vascular tissue [20–22].

In the present study, we determined the ability of 4-HHE to induce Nrf2-mediated antiatherogenic genes such as HO-1 and to prevent oxidative stress-induced cytotoxicity in vascular endothelial cells, in order to elucidate the biological mechanisms responsible for the antiatherogenic effects of *n*-3 PUFAs.

2. Materials and methods

2.1. Reagents

MCDB 131 medium, L-glutamine, and peroxidase-linked anti-mouse antibody were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from Biowest (Miami, FL). Basic fibroblast growth factor was purchased from Kaken Pharmaceutical (Tokyo, Japan). 4-HHE and 4-hydroxy nonenal (4-HNE) were purchased from Cayman (Ann Arbor, MI). *Tert*-butyl hydroperoxide (tBHP) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO). Anti-Nrf2 antibody (H-300), anti-lamin A/C antibody and peroxidase-linked anti-rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB374) was from Millipore (Billerica, MA). Anti-HO-1 antibody was from Assay Design (Ann Arbor, MI).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultured according to a previously reported method [23]. 4-HHE and 4-HNE were dissolved in ethanol, and further dissolved in serum-containing medium at the final desired concentration. The control medium containing ethanol was prepared in a similar manner.

2.3. MTT measurement of cell viability

HUVECs at the 4th passage were seeded on 24-well type I-collagen-coated plates. Confluent cells were treated with different concentrations (5, 10, 25 and 50 μM) of 4-HHE or 4-HNE for 24 h. Cell viability was determined by the conventional MTT reduction assay. MTT is a tetrazolium salt cleaved to formazan by the mitochondrial respiratory chain enzyme, succinate dehydrogenase. After treatment with 4-HHE or 4-HNE, cells were incubated with the MTT solution (0.5 mg/ml) in culture medium for 3 h. The culture medium was then removed, the formazan product was solubilized by dimethyl sulfoxide, and the absorbance at 577 nm was measured using a microplate reader. In order to determine the preventive effect of 4-HHE or 4-HNE on tBHP-induced cell toxicity, cells were pretreated with 4-HHE (5 and 10 μM) or 4-HNE (5 μM) for 12 h, washed with phosphate-buffered saline (PBS), and exposed to tBHP (250 or 500 μM) for 6 h. Cell viability was determined using the MTT assay, as above.

2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted from the cells using a Total RNA Mini Kit (Bio-Rad, Hercules, CA). Single-strand cDNA was synthesized from 0.5 μg of total RNA using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Quantitative analyses of HO-1, NQO1 and Nrf2 mRNAs were performed by real-time PCR using the ABI 7500 Fast real-time PCR system (Applied Biosystems, Japan). Premix Ex Taq (Takara Bio) and Assay-on-Demand, Gene Expression Products [Hs01110250_m1 for HO-1, Hs00168547_m1 for NQO1, Hs00232352_m1 for Nrf2 and Hs02387368_g1 for RPS18 (Applied Biosystems, Foster City, CA)] were used for quantitative real-time PCR analysis. All the quantitative data were normalized to the expression level of ribosomal protein S18 (RPS18).

2.5. Western blotting analysis

Whole cell lysate from HUVECs was prepared in RIPA buffer (Thermo Scientific, Rockford, IL) containing protease inhibitor (Thermo Scientific). Nuclear lysate was prepared using a Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA), according to the manufacturer's protocol. Whole cell and nuclear lysates were denatured by boiling in SDS sample buffer (Thermo Scientific), resolved by SDS–PAGE and then transferred to nitrocellulose membrane by electroblotting. Blots were then incubated with a rabbit anti-HO-1 primary antibody, a rabbit anti-Nrf2 primary antibody, a rabbit anti-lamin A/C primary antibody, or a mouse anti-GAPDH primary antibody plus a horseradish peroxidase-linked secondary antibody, and detected by chemiluminescence using an ImageQuant LAS 4000mini system (GE Healthcare, Japan).

2.6. Nrf2 DNA binding assay

Nrf2 activation was assayed using Active Motif's (Carlsbad, CA) ELISA-based transactivation TransAM kit, following the manufacturer's protocol. Nrf2 from nuclear lysate, which specifically binds to its consensus oligonucleotide, was analyzed colorimetrically by a spectrophotometer at 450 nm.

2.7. Transfection with small interfering (si)RNA

HUVECs were plated in type I-collagen-coated plates until 80–90% confluency. siRNA against Nrf2 was used to silence Nrf2 (On-TARGET plus SMARTpool Reagent; Thermo Scientific). A control siRNA was also used (On-TARGET plus Non-targeting siRNA #1, Thermo Scientific). HUVECs were transfected with 20 nM of Nrf2 siRNA or control siRNA using DharmaFECT 1 siRNA Transfection Reagent (Thermo Scientific) and incubated for 24 h in medium containing 2% FBS, after which the medium was refreshed. After a further 24-h incubation, HUVECs were stimulated with 4-HHE or 4-HNE. The silencing effect of Nrf2 was confirmed by real-time PCR and Western blotting analyses.

2.8. Statistical analysis

Data are presented as means ± SE. Differences between more than three groups were analyzed by two-tailed multiple *t*-tests

with Bonferroni correction. Comparisons between two groups were analyzed using two-tailed Student *t*-tests. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Cytotoxicity of 4-HHE and 4-HNE in HUVECs

HUVECs were treated with various concentrations (5, 10, 25 and 50 μM) of 4-HHE or 4-HNE for 24 h, and cytotoxicity was determined by MTT assay. 4-HHE at 50 μM caused significant cell death (17%), as did 4-HNE (25 μM, 17%; 50 μM, 99%), while 4-HHE (5–25 μM) and 4-HNE (5–10 μM) had no effects on cell viabilities.

3.2. Effects of 4-HHE and 4-HNE on mRNA and protein expressions of HO-1 or NQO1 in HUVECs

To determine if 4-HHE or 4-HNE induced the antioxidative enzyme HO-1, HUVECs were treated with 5 μM of each reagent for 2, 6 or 24 h. 4-HHE, as well as 4-HNE, caused a maximal increase in HO-1 mRNA expression after 6 h (Fig. 1A). Treatment for 6 h also dose-dependently increased the mRNA and intracellular protein expressions of HO-1 at concentrations of 1–10 μM (Fig. 1B and D). 4-HHE also significantly increased the mRNA expression of NQO1, a different ARE-responsive gene (Fig. 1C).

3.3. Activation of Nrf2 by 4HHE and 4-HNE

To explore the role of transcription factor Nrf2 activation in mediating the stimulation of HO-1 expression, translocation of Nrf2 to the nucleus was evaluated by Western blotting analysis of Nrf2 in the nuclear fraction. As shown in Fig. 2A, treatment with 4-HHE (5 μM) or 4-HNE (5 μM) for 6 h increased Nrf2 in the nuclear lysate. Fig. 2B shows Nrf2 activation induced by 4-HHE or 4-HNE. The binding activities of Nrf2 to its consensus oligonucleotide were significantly increased by stimulation with 4-HHE or 4-HNE. However, neither reagent had any effect on Nrf2 mRNA expression (Fig. 2C).

3.4. Effects of 4-HHE and 4-HNE on HO-1 expression in Nrf2 knockdown HUVECs

To determine the role of Nrf2 in the induction of HO-1 by 4-HHE or 4-HNE, HUVECs were transfected with either Nrf2 siRNA to decrease Nrf2 expression, or with scrambled siRNA as a control. Nrf2 downregulation after treatment with Nrf2 siRNA was confirmed by

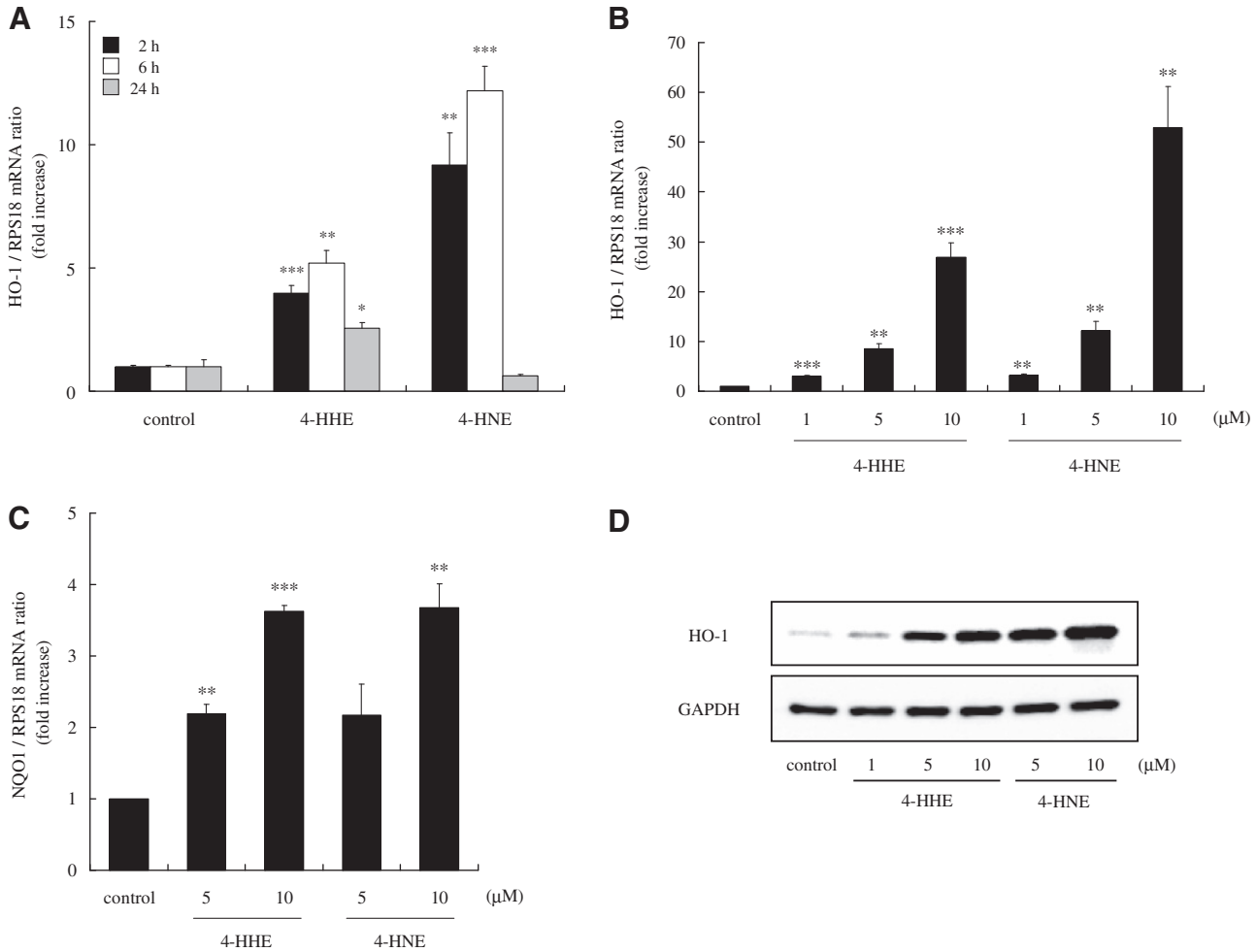

Fig. 1. Effects of 4-HHE and 4-HNE on HO-1 or NQO1 expression in HUVECs. HUVECs were incubated in the presence of 4-HHE or 4-HNE (1–10 μM). (A, B, and C) Total RNA was extracted after incubation for 2–24 h and the relative mRNA expressions of HO-1 or NQO1 were analyzed quantitatively using real-time RT-PCR. The results were normalized to RPS18 and expressed as fold increase over control. Values represent the means ± SE of three or four experiments. (D) Total cell lysates from HUVECs after incubation for 6 h were resolved by SDS–PAGE and subjected to Western blotting analyses with HO-1 antibody, or GAPDH antibody as an endogenous control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with each corresponding control.

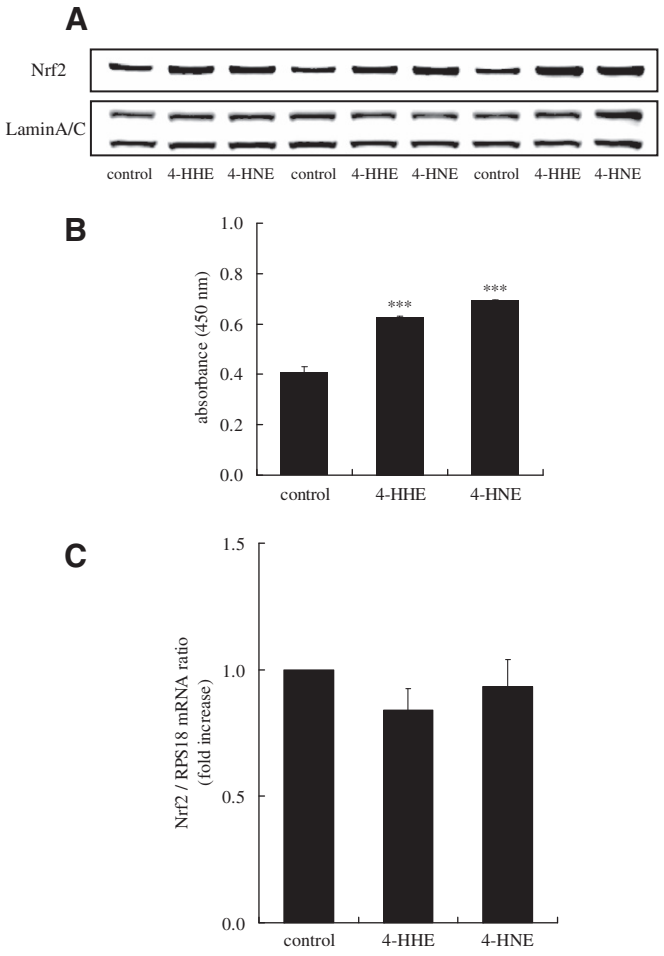

Fig. 2. Effects of 4-HHE and 4-HNE on Nrf2 activation in HUVECs. HUVECs were incubated in the presence of 4-HHE (5 μM) or 4-HNE (5 μM) for 6 h. (A) Nuclear lysates from HUVECs were resolved by SDS–PAGE and subjected to Western blotting analyses with Nrf2 antibody, or lamin A/C antibody as an endogenous control. (B) Analysis of the binding of Nrf2 in nuclear lysates to its consensus oligonucleotide was performed using the ELISA-based TransAM Nrf2 kit. Values are expressed as the means ± SE of three experiments. (C) Total RNA was extracted and the relative mRNA expression of Nrf2 was analyzed quantitatively using real-time RT-PCR. Results were normalized to RPS18 and expressed as fold increase over control. Values are expressed as the means ± SE of four experiments. $^{***}P < 0.001$, compared with control.

quantitative RT-PCR and Western blotting analyses. The expression of Nrf2 mRNA in cells treated with Nrf2 siRNA was reduced by approximately 80% (Fig. 3A), while Nrf2 protein expression in whole cell lysate was also markedly suppressed (Fig. 3B). As shown in Fig. 3C, the increases in HO-1 mRNA expression caused by 4-HHE (5 and 10 μM) or 4-HNE (5 μM) were significantly suppressed by Nrf2 knockdown. Similarly, knockdown of Nrf2 also reduced 4-HHE or 4-HNE-induced HO-1 protein expression (Fig. 3D).

3.5. Preventive effects of 4-HHE and 4-HNE on oxidative stress-induced cell toxicity

4-HHE and 4-HNE increased expression of the antioxidant enzyme HO-1 through the activation of Nrf2, and their cytoprotective effects against tBHP-induced oxidative stress were therefore investigated in HUVECs. Fig. 4 shows cell viability assessed by MTT assay 6 h after the induction of oxidative stress by tBHP. Treatment with tBHP (250 and 500 μM) caused significant cytotoxicity, while pretreatment with 4-HHE or 4-HNE significantly protected against tBHP-induced cytotoxicity at concentrations from 5 to 10 μM.

4. Discussion

4-HHE is derived from the oxidation of *n*-3 PUFAs such as DHA or EPA, and has been considered to be a toxic lipid peroxidation product similar to 4-HNE, a peroxidation product of *n*-6 PUFAs. 4-HHE forms adducts on DNA, protein, or phospholipids, and recent studies have demonstrated the presence of 4-HHE adducts in atherosclerotic lesions [24] and diabetic retinas [25]. 4-HHE has also been reported to induce cytotoxicity and apoptosis in several cell types, including epithelial and endothelial cells. In rat prostate endothelial YPEN-1 cells, 20 μM 4-HHE caused cell death by inducing apoptotic Bax, coupled with a decrease in anti-apoptotic Bcl-2, and the induction of reactive oxygen species, nitric oxide or peroxynitrite in serum-free media [26]. Choudhary et al. also reported that 4-HHE at concentrations over 20 μM induced apoptosis in retinal pigmented epithelial [27] or lens epithelial cells [28]. The results of the current study confirmed that 4-HHE, as well as 4-HNE, caused cell death at higher concentrations (25–50 μM). However, it is plausible that increased levels of 4-HHE associated with the intake of fish oils such as DHA or EPA may play an important atheroprotective role at lower concentrations. Siow et al. proposed that low concentrations of 4-HNE (<10 μM), an *n*-6 PUFA oxidation product, acted as an atheroprotective molecule by enhancing Nrf2-mediated antioxidant gene expression [29]. More recently, intravenous administration of 4-HNE was shown to protect against cardiac ischemia–reperfusion injury in mice via an Nrf2-dependent pathway [30].

In the present study, we demonstrated that 4-HHE, as well as 4-HNE, induced HO-1 expression at comparatively low concentrations in vascular endothelial cells. We also observed that 4-HHE increased Nrf2 translocation and DNA binding to the consensus sequence. Knockdown of Nrf2 with siRNA also reduced the expression of HO-1 induced by 4-HHE, while pretreatment with 4-HHE prevented oxidative stress-induced cytotoxicity. This is the first report to demonstrate activation of Nrf2 by 4-HHE, leading to induction of the adaptive antioxidative response gene and consequent cell protection against oxidative stress, although the Nrf2-mediated HO-1 expression was not shown to be critical for the preventive effect of 4-HHE on tBHP-induced cytotoxicity. However, inhibition of HO-1 activity in endothelial cells has previously been reported to attenuate the prevention of oxidative stress-induced cytotoxicity accompanied by HO-1 induction [31,32], thus supporting the suggestion that 4-HHE could prevent tBHP-induced cell death through the induction of HO-1.

Recent studies have already demonstrated that HO-1 plays an important role in preventing atherogenesis *in vivo*. HO-1 prevents plaque instability by impeding lipid deposition and necrotic core growth and by prolonging survival of vascular smooth muscle cells in the fibrous cap [20]. Juan et al. [21] reported that overexpression of HO-1 in vascular cells attenuated the development of atherosclerosis in apoE-deficient mice, and True et al. [22] also demonstrated that HO-1 protected against intravascular thrombosis associated with oxidative damage to the endothelium in a carotid artery injury model of HO-1 deficient mice. The increased HO-1 expression induced by 4-HHE as an *n*-3 PUFA peroxidation product may thus partly explain the cardioprotective effects of *n*-3 PUFAs, though further studies are needed to investigate the ability of 4-HHE to prevent atherogenesis *in vivo*.

The biological mechanisms whereby 4-HHE affects Nrf2 activation remain unclear, though Nrf2 silencing showed that the 4-HHE-induced HO-1 increase depended on Nrf2 activation. Recent studies in HUVECs suggest that 4-HNE can directly modify cysteine

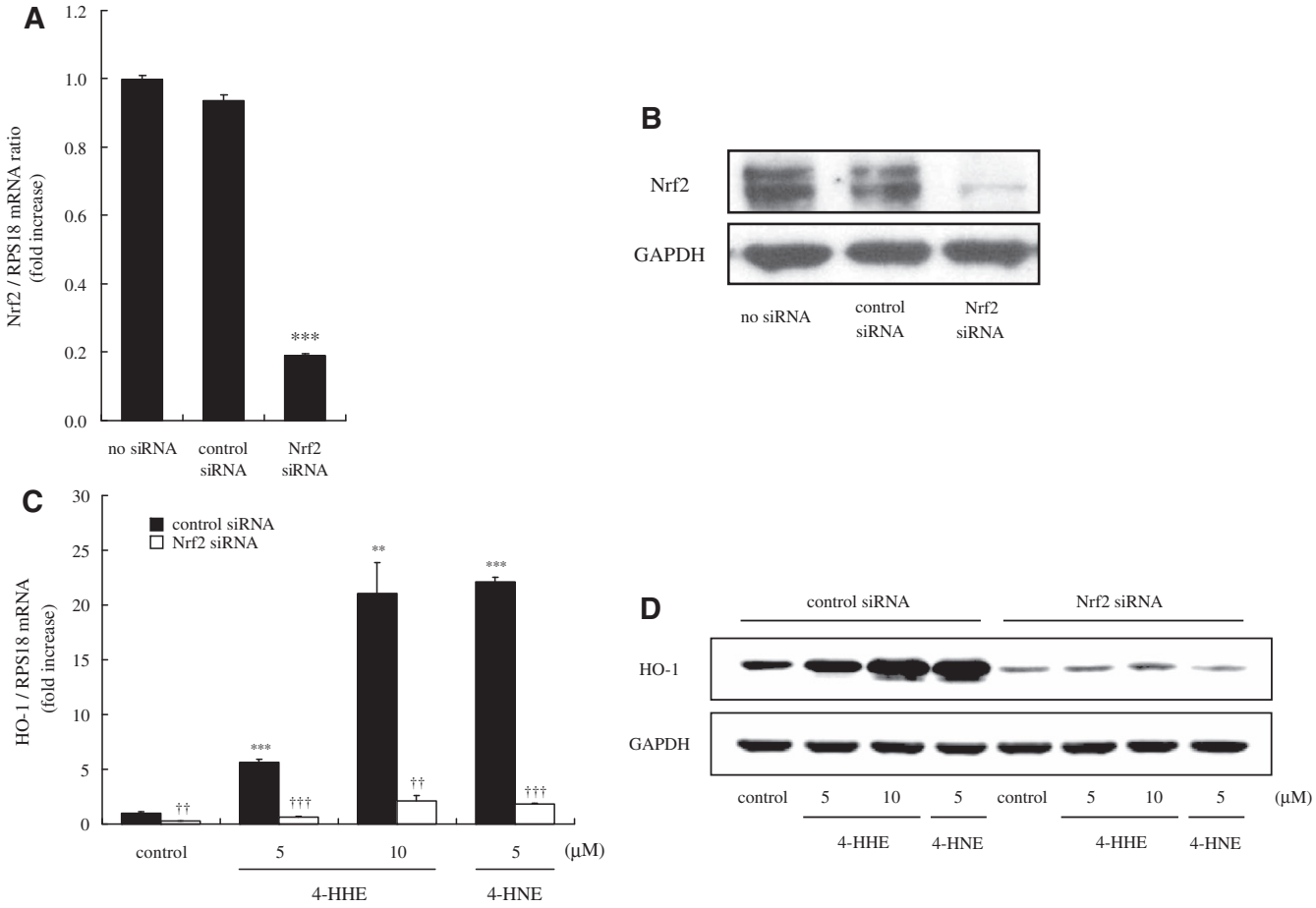

Fig. 3. Effects of 4-HHE and 4-HNE on the expression of HO-1 in Nrf2 knockdown HUVECs. (A and B) HUVECs were treated with Nrf2 siRNA or control siRNA, and incubated for 48 h. (A) Total RNA was extracted and the relative mRNA expression of Nrf2 was analyzed quantitatively using real-time RT-PCR. Results were normalized to RPS18 and expressed as fold increase over no siRNA control. Values are expressed as the means ± SE of four experiments. (B) Whole cell lysates from HUVECs were resolved by SDS–PAGE and subjected to Western blotting analyses with Nrf2 antibody, or GAPDH antibody as an endogenous control. (C and D) HUVECs were transfected with siRNA targeted against Nrf2 or control siRNA. After 48 h, the cells were incubated with 4-HHE (5 and 10 μM) or 4-HNE (5 μM) for a further 6 h. (C) Total RNA was extracted and the relative mRNA expression of HO-1 was analyzed quantitatively using real-time RT-PCR. Results were normalized to RPS18 and expressed as fold increase over control treated with control siRNA. Values are expressed as the means ± SE of three experiments. (D) Whole cell lysates from HUVECs were resolved by SDS–PAGE and subjected to Western blotting analyses with HO-1 antibody, or GAPDH antibody as an endogenous control. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control cells treated with control siRNA, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$, compared with the corresponding cells treated with control siRNA.

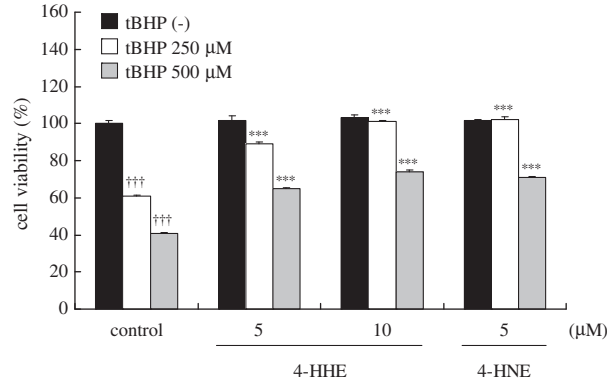

Fig. 4. Preventive effects of 4-HHE and 4-HNE on tBHP-induced cytotoxicity in HUVECs. HUVECs were pretreated in the presence of 4-HHE (5 and 10 μM) or 4-HNE (5 μM) for 12 h, and then stimulated with tBHP (250 and 500 μM) for 6 h. Cell viability was determined by MTT assay. Values are expressed as the percentage of cell survival. Values represent the means ± SE of three experiments. $^{***}P < 0.001$, compared with tBHP-treated control, $^{\dagger\dagger\dagger}P < 0.001$, compared with tBHP-untreated control.

residues on Keap1, leading to activation of Nrf2 [33]. 4-HNE has also been reported to stimulate reactive oxygen species generation from mitochondria of bovine aortic endothelial cells [34], activating Nrf2. Further studies are therefore also needed to elucidate the molecular mechanisms responsible for the activation of Nrf2 by 4-HHE.

5. Conclusions

This study demonstrated that 4-HHE is able to stimulate HO-1 expression through the activation of Nrf2, and shows a preventive effect against oxidative stress-induced cytotoxicity in vascular endothelial cells, providing a partial explanation for the cardioprotective effects of *n*-3 PUFAs.

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