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4-Hydroxy hexenal derived from dietary n-3 polyunsaturated fatty acids induces anti-oxidative enzyme heme oxygenase-1 in multiple organs

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ABSTRACT

It has recently been reported that expression of heme oxygenase-1 (HO-1) plays a protective role against many diseases. Furthermore, n-3 polyunsaturated fatty acids (PUFAs) were shown to induce HO-1 expression in several cells *in vitro*, and in a few cases also *in vivo*. However, very few reports have demonstrated that n-3 PUFAs induce HO-1 *in vivo*.

In this study, we examined the effect of fish-oil dietary supplementation on the distribution of fatty acids and their peroxidative metabolites and on the expression of HO-1 in multiple tissues (liver, kidney, heart, lung, spleen, intestine, skeletal muscle, white adipose, brown adipose, brain, aorta, and plasma) of C57BL/6 mice. Mice were divided into 4 groups, and fed a control, safflower-oil, and fish-oil diet for 3 weeks. One group was fed a fish-oil diet for just 1 week. The concentration of fatty acids, 4-hydroxy hexenal (4-HHE), and 4-hydroxy nonenal (4-HNE), and the expression of HO-1 mRNA were measured in the same tissues.

We found that the concentration of 4-HHE (a product of n-3 PUFAs peroxidation) and expression of HO-1 mRNA were significantly increased after fish-oil treatment in most tissues. In addition, these increases were paralleled by an increase in the level of docosahexaenoic acid (DHA) but not eicosapentaenoic acid (EPA) in each tissue. These results are consistent with our previous results showing that DHA induces HO-1 expression through 4-HHE in vascular endothelial cells. In conclusion, we hypothesize that the HO-1-mediated protective effect of the fish oil diet may be through production of 4-HHE from DHA but not EPA in various tissues.

1. Introduction

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme with anti-oxidant and anti-inflammatory properties [1–4]. This enzyme metabolizes heme to carbon monoxide (CO), Fe^{2+}, and biliverdin. CO increases the level of cellular cyclic guanosine monophosphate (c-GMP), which regulates ion channel conductance, vasodilation, and cellular apoptosis [5]. Biliverdin is converted to bilirubin (a potent free radical scavenger) by biliverdin reductase [6]. HO-1 is a well-known target gene of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor that is regulated by the Kelch-like erythroid-cell-derived protein with CNC homology associated protein 1 (Keap1) pathway [3,7]. Nrf2 regulates the environmental stress response by activating the expression of genes for anti-oxidative and detoxification enzymes against a variety of stressors including environmental pollutants (such as electrophiles), oxidative stress, immunotoxicants, and inflammation [7–9].

An excess of reactive oxygen species (ROS) or free radicals caused by high fat, high sugar diets is responsible for critical damage to DNA and accelerates the progression of several diseases such as non-alcoholic steatohepatitis, type 2 diabetes mellitus, and its complications. Recently, we reported that docosahexaenoic acid (DHA) and its peroxidation product, 4-HHE, activate the Nrf2 HO-1 pathway in human umbilical vein endothelial cells (HUVECs) [10,11]. In addition, DHA and 4-HHE protect cells from oxidative stress in HUVECs and 3T3-L1 adipocytes in Nrf2-dependent manner [11,12]. It has also been reported that DHA induces Nrf2-mediated HO-1 expression in human smooth muscle cells isolated

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from small pulmonary artery or endothelial cells [13,14], and a few reports have demonstrated that HO-1 is regulated by n-3 PUFAs *in vivo* [15–18]. Therefore, we examined the effect of fish-oil dietary supplementation on HO-1 expression and the distribution of fatty acids and their peroxidation products in multiple organs (relative to the aorta) of C57BL/6 mice, thus establishing the relationship of DHA and 4-HHE concentrations to HO-1 expression in multiple organs.

2. Materials and methods

2.1. Animals and experimental procedure

Eight-week-old male C57BL/6 mice were purchased from CLEA Japan Inc. Sixteen mice were divided into 4 groups, and fed a control diet (#110700, AIN-93G, Dyets Inc., Bethlehem, PA), safflower-oil diet (#112245), and fish-oil diet (#112246) for 3 weeks (Lipids component: Supplementary Chart S1). One group was fed the fish-oil diet for 1 week. All animal experimentation was approved by the committee on Animal Research of Shiga University of Medical Science (Permit Number: 2013-2-3H). After 1 or 3 weeks of the dietary intervention, C57BL/6 mice were sacrificed by injection of an overdose (160 mg/kg) of pentobarbital sodium salt. Plasma was obtained by centrifuging whole blood at 700*g* at 4 °C for 5 min. Liver, kidney, heart, lung, spleen, intestine, skeletal muscle, white adipose, brown adipose, brain, and aorta were dissected, immediately freeze-clamped by liquid nitrogen, and stored at under −80 °C until lipid extraction and mRNA extraction. To prepare them for lipid extraction, the tissues in liquid nitrogen were pulverized into a fine powder using a Cryo Press disruptor (Microtec Co., Ltd., Chiba, Japan). This fine powder was weighed on an electronic balance ME235 (Sartorius AG, Göttingen, Germany), homogenized in 490 μL of chloroform/methanol (1:1, v/v) and 10 μL dibutylhydroxytoluene solution (10 mg/mL in ethanol), and incubated at 36 °C for 1 h [19]. The resulting solution was used for measuring the concentration of fatty acids, 4-HHE, and 4-hydroxy nonenal (4-HNE).

2.2. Quantitative analysis of fatty acids levels in biological samples

Fatty acids were measured in biological samples using a modified liquid chromatography–tandem mass spectrometry (LC/MS/MS) procedure [20]. Standard solutions of myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA; Cayman Chemical Co., Ann Arbor, MI) were used for calibration curves. The following solutions were transferred to glass tubes: 20 μL of supernatant of homogenized tissue, serum, or phosphate buffered saline (used for calibration curves), internal standard solution (10 μg/mL; [$^2H_5$]-EPA and [$^2H_5$]-DHA [Cayman Chemical Co.]), and 200 μL of acetontrile/6N HCl (90/10, v/v). The tubes were capped, incubated at 100 °C for 45 min, and cooled to room temperature. Then, 200 μL of methanol/10N NaOH (90/10, v/v) was added; the tubes were capped, incubated at 100 °C for 45 min, and cooled to room temperature. Liquid/liquid extraction was done using ethyl acetate. The upper layer was evaporated under a stream of nitrogen gas at 36 °C, reconstituted with 40 μL of acetonitrile, and injected into an optimized LC/MS/MS system. LC was performed using an ACQUITY UPLC (Waters, Milford, MA) and an API4000 triple quadrupole tandem mass spectrometer (AB Sciex, Foster City, CA). A YMC-Triart C18 analytical column (2.0 × 100 mm, particle size 1.9 μm; YMC Co., Ltd., Kyoto, Japan) was used for separating the fatty acids. An atmospheric pressure chemical ionization source was operated in negative ionization and selected reaction monitoring (SRM) mode. The SRM transitions for precursor to product ion were as follows: myristic acid, *m/z* 277-277; palmitic acid, *m/z* 255-255; palmitoleic acid, *m/z* 253-253; stearic acid, *m/z* 283-283; oleic acid, *m/z* 281-281; linoleic acid, *m/z* 279-279; α-linolenic acid, *m/z* 277-277; γ-linolenic acid, *m/z* 277-277; dihomo-γ-linolenic acid, *m/z* 305-305; arachidonic acid (AA), *m/z* 303-259; eicosapentaenoic acid (EPA), *m/z* 301-257; docosapentaenoic acid (DPA), *m/z* 329-329, and docosahexaenoic acid (DHA), *m/z* 327-283. Other parameters were adjusted to optimum values.

2.3. Quantitative analysis of 4-HHE and 4-HNE in biological samples

4-HHE and 4-HNE in biological samples were quantitatively analyzed using a modified LC/MS/MS procedure [21,22]. Standard solutions of 4-HHE and 4-HNE (Cayman Chemical Co.) were used for calibration curves. The following solutions were transferred to glass tubes: 200 μL of supernatant of homogenized tissues, serum, or phosphate buffered saline (used for calibration curves), and internal standard solution (100 ng/mL; [$^2H_3$]-4-HNE (Cayman Chemical Co.). Solid phase extraction was done using a mixed-mode anion exchange solid-phase extraction (SPE) cartridge (Oasis MAX; Waters). 4-HHE and 4-HNE were derivatized by incubation with t-butylhydroxytoluene solution and 600 μL of acidified cyclohexanedione (CHD) reagent at 60 °C for 1 h. After the tubes cooled down to room temperature, the derivatized 4-HHE and 4-HNE were extracted using a reversed-phase SPE cartridge (Oasis HLB; Waters). The eluate from the SPE cartridge was evaporated to dryness under a stream of nitrogen gas at 36 °C. An aliquot of the reconstituted extract was injected into an optimized LC/MS/MS system. LC/MS/MS analysis was performed as described previously for the measurement of fatty acids. An ACQUITY CSH C18 column (2.0 × 100 mm, particle size 1.7 μm; Waters) was used for separating 4-HHE and 4-HNE. Electrospray ionization (ESI) was carried out with the API4000 operating in the positive ionization and SRM mode. The SRM transitions for CHD-derivatized 4-HHE and 4-HNE respectively, were *m/z* 284-216 and *m/z* 326-216. Other parameters were adjusted to optimum values.

2.4. mRNA extraction and real-time PCR analysis

Total RNA was extracted from each tissue using TRIzol reagent (Life Technologies, Carlsbad, CA). Tissues were pulverized in liquid nitrogen using a Cryo Press and subjected to RNA extraction. Single-stranded cDNA was synthesized from 1 μg of total RNA using a Prime Script RT Reagent Kit (Takara Bio, Shiga, Japan), and endogenous genomic DNA was degraded by DNase I (Life Technologies). Real-time RT-PCR reactions were carried out in SYBR Green PCR master mix (Life Technologies) and quantitative analyses of HO-1 mRNA were performed by real-time PCR using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Quantitative data were normalized to the expression level of ribosomal protein S18 (RPS18). Real-time PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For real-time PCR, the HO-1 primers were sense 5′-CGAAACAAGCAGAACCCAGTCT-3′ and antisense 5′-AGCCCTTCGGT GCAGCT-3′. The RPS18 primers were sense 5′-′TGGCTGAACGCC ACTTGTC-3′ and antisense 5′-TTCCGATAACGAACGAGAGACTCT-3′ (Sigma–Aldrich Corporation, St. Louis, MO, USA).

2.5. Statistical analysis

Data are presented as mean ± SD. Differences between more than three groups were analyzed by two-tailed multiple *t*-test with Bonferroni correction. Comparisons between two groups were

analyzed using a two-tailed Student's *t*-test. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Concentration of fatty acid, 4-HHE and 4-HNE in plasma

Absolute concentrations of 13 fatty acids, 4-HHE, and 4-HNE were measured using LC/MS/MS. Plasma EPA, DPA, and DHA levels were significantly higher at both 1 and 3 weeks in the fish-oil diet groups than the control and safflower-oil groups (Fig. 1A). On the other hand, some of the fatty acids (AA, oleic acid, and linoleic acid) were significantly decreased in the fish-oil diet group. Furthermore, 4-HHE level was significantly higher (Fig. 1B) while 4-HNE level was significantly lower (Fig. 1C) at both 1 and 3 weeks in the fish-oil diet group.

3.2. Concentration and content of EPA, DHA, and AA in each tissue

Absolute concentrations of EPA, DHA, and AA in each tissue were measured using the same method of LC/MS/MS analysis used above for plasma. Both concentration and content of EPA in all tissues were significantly higher in the fish-oil groups than the control and safflower-oil groups (Fig. 2A and D). Both DHA concentration and DHA content were also significantly higher in every tissue except skeletal muscle and brain (Fig. 2B and E). In contrast, AA concentration and AA content were lower in every tissue except brown adipose and white adipose tissues of the fish-oil group (Fig. 2C and 2F). In addition, concentration of linoleic acid, a major component of safflower oil, was significantly higher in the safflower-oil group than the others (Supplementary Fig. S1).

3.3. Concentration of 4-HHE and 4-HNE in each tissue

The concentrations of 4-HHE (an end product of n-3 PUFAs peroxidation) as well as 4-HNE (an end product of n-6 PUFAs peroxidation) were measured in multiple tissues of the different dietary groups. The 4-HHE concentration in tissues except for brain was significantly higher in the fish oil group than in the control and safflower-oil diet groups (Fig. 3A). In contrast, the 4-HNE concentration remained unchanged or decreased in response to the fish-oil diet (Fig. 3B).

3.4. Expression of HO-1 mRNA in each tissue

Expression of HO-1 mRNA after treatment with fish-oil was analyzed in multiple organs. HO-1 mRNA expression was significantly increased in all tissues except spleen and brain (Fig. 4). Expression of HO-1 in every group was higher in the spleen than in other tissues.

4. Discussion

Our study had two important findings. First, the concentration of 4-HHE, a product of n-3 PUFAs peroxidation, but not the concentration of 4-HNE, was significantly increased after fish-oil treatment in all tissues. Second, the expression of HO-1 mRNA was also significantly increased after fish-oil treatment.

With regard to the significant increase we noted in 4-HHE in all tissues after fish oil treatment for 1 and 3 weeks, a previous report showed that plasma 4-HHE levels were increased following supplementation with 800 or 1600 mg/day of DHA in human volunteers [23], and two reports showed increased levels of protein

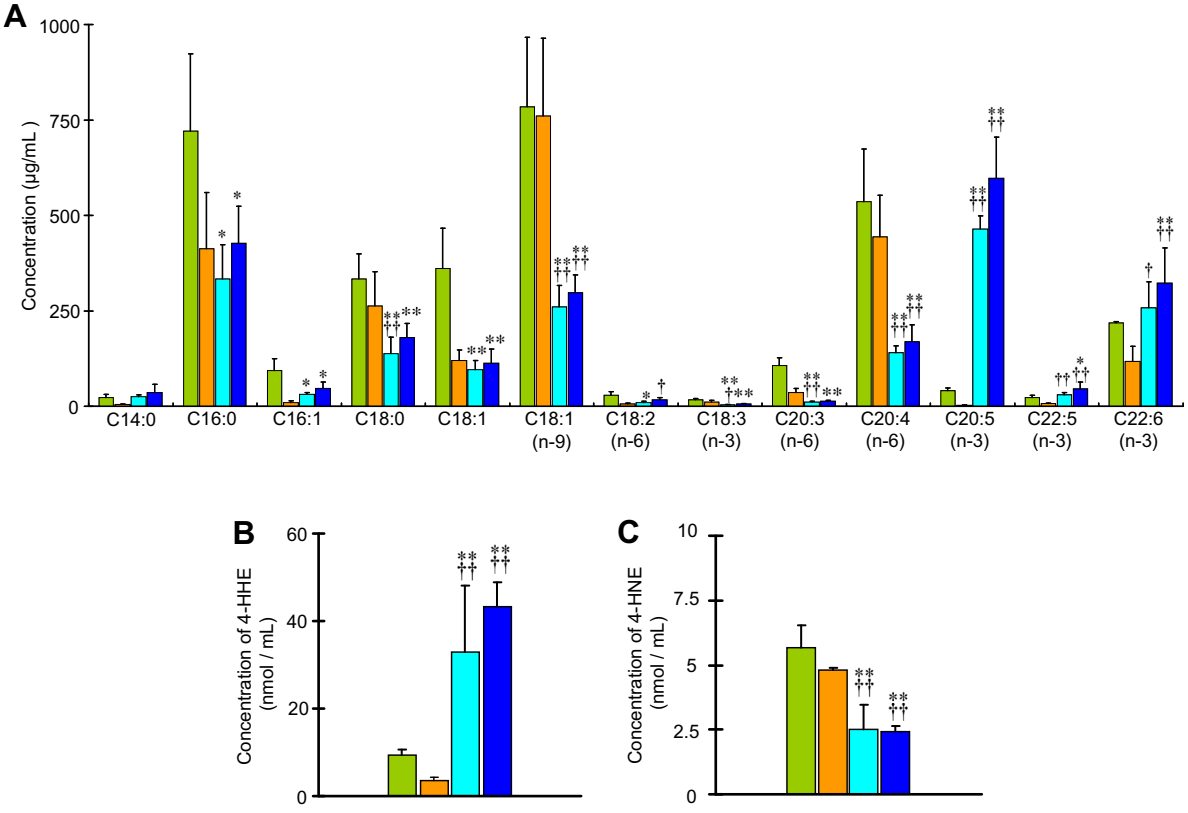

Fig. 1. Plasma concentration of fatty acids, 4-HHE and 4-HNE in C57BL/6 mice. Green bar; control diet with 3 weeks, Orange bar; safflower oil diet with 3 weeks Light blue bar; fish oil diet with 1 week, Dark blue bar; fish oil diet with 3 weeks.(A) Plasma concentration of fatty acids. (B) Plasma concentration of 4-HHE. (C) Plasma concentration of 4-HNE were measured using LC/MS/MS. Each value represents the mean ± SD of four animals. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control diet group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with safflower oil diet group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

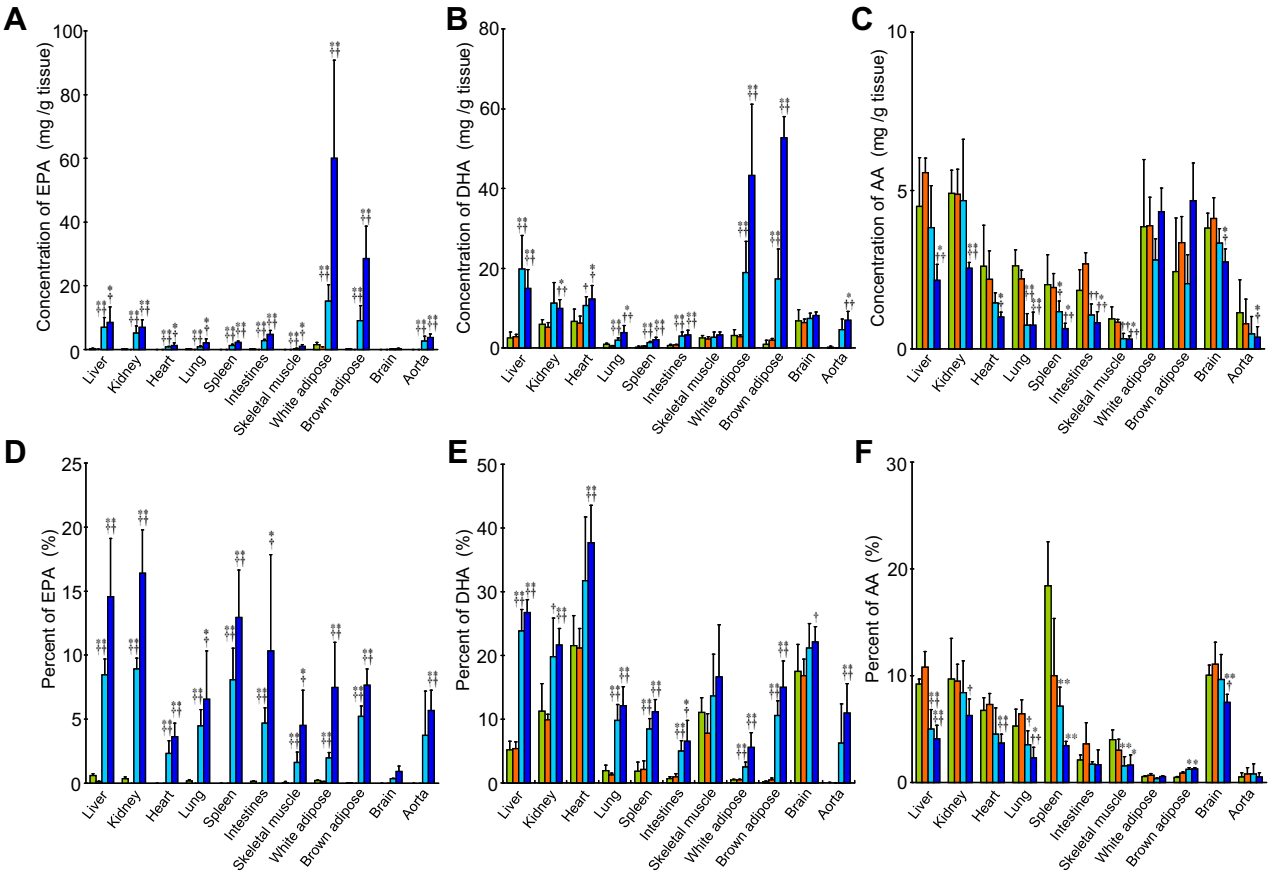

Fig. 2. Concentration and composition of EPA, DHA and AA in multiple organs in C57BL/6 mice. Green bar; control diet with 3 weeks, Orange bar; safflower oil diet with 3 weeks Light blue bar; fish oil diet with 1 week, Dark blue bar; fish oil diet with 3 weeks.(A) Concentration of EPA. (B) Concentration of DHA. (C) Concentration of AA were measured using LC/MS/MS. (D) Composition of EPA. (E) Composition of DHA. (F) Composition of AA were calculated by total fatty acids concentration. Published data applicable to the aorta [11] are also included for comparison. Each value represents the mean ± SD of four animals. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control diet group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with safflower oil diet group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adducts of 4-HHE in heart and liver of animals fed an n-3 PUFA diet [24,25]. In addition, the concentration of 4-HHE was closely related to the fatty acid composition of DHA in all tissues we analyzed. DHA content was higher in the order of heart greater than liver, liver greater than kidney, and kidney greater than skeletal muscle. The concentration of 4-HHE was similarly higher in these tissues, and thus DHA content was positively correlated with 4-HHE concentration ($r = 0.716$). By contrast, the relationship of EPA content and 4-HHE levels in these tissues was quite different. Furthermore, in some tissues, the increase in EPA content in fish-oil groups was relatively small even though 4-HHE was strongly detected. Thus, EPA content was less related to 4-HHE levels ($r = 0.376$). These data suggest that 4-HHE is preferentially oxidized from DHA, not EPA.

With regard to our finding that dietary fish-oil markedly increases HO-1 mRNA expression in all single tissues we analyzed except spleen and brain, recent studies have shown that dietary fish-oil can increase the expression of Nrf2 target genes including the HO-1 gene in the kidney and liver [16,19], and that DHA inhibits NF-κB-mediated endothelial inflammation through the Nrf2-HO-1 pathway [26]. Our results also indicate that dietary fish-oil induces the anti-oxidative enzyme HO-1 in multiple organs but not in brain and spleen. No increase in HO-1 expression in the brain is probably due to the smaller increase in 4-HHE concentration in the brain compared with that in other tissues. In contrast, both levels of DHA and 4-HHE were significantly increased in the spleen. Spleen has the highest HO-1 expression of any organ [27–29]. Because heme is known as the inducer of HO-1 expression, we speculate that HO-1 expression in the spleen is regulated mainly by heme rather than 4-HHE [30].

Our recent studies demonstrate that 4-HHE, a product of DHA peroxidation, activates Nrf2-mediated HO-1 expression in 3T3-L1 adipocytes and HUVECs, and also in the aortic tissue of mice [10–13]. In this study, the increase in 4-HHE concentration and HO-1 mRNA expression were closely associated in all tissues except spleen (the relation between 4-HHE concentration and HO-1 mRNA expression being $r = 0.608$). Liver, kidney, and heart tissues with high 4-HHE concentrations also showed high HO-1 mRNA expression, while adipose tissue with lower 4-HHE concentrations showed lower HO-1 mRNA expression. Therefore, the relationship of DHA content to 4-HHE concentration and HO-1 mRNA expression in these tissues suggest that 4-HHE derived from DHA may induce HO-1 mRNA expression through activation of Nrf2.

Most previous reports demonstrate that 4-HHE induces cytotoxic and negative effects in cells such as rat primary neurons, YPEN-1 prostatic endothelial cells, lens epithelial cells, and U937 lymphocytes [31–33]. In YPEN-1 cells, 4-HHE increases pro-apoptotic Bax levels, decreases Bcl-2 level, and induces ROS and peroxynitrite production [32]. Another report also shows that 4-HHE induces activation of the NF-κB pathway in YPEN-1 cells, resulting in elevated expression of inducible nitric oxide synthases (iNOs) and nitric oxide production [34]. By contrast, we observed that fish oil exerts beneficial effects in mice through the activation of Nrf2 by 4-HHE in HUVECs and the aorta. The much higher concentration of 4-HHE in previous studies (μM) [32–34] in contrast to the

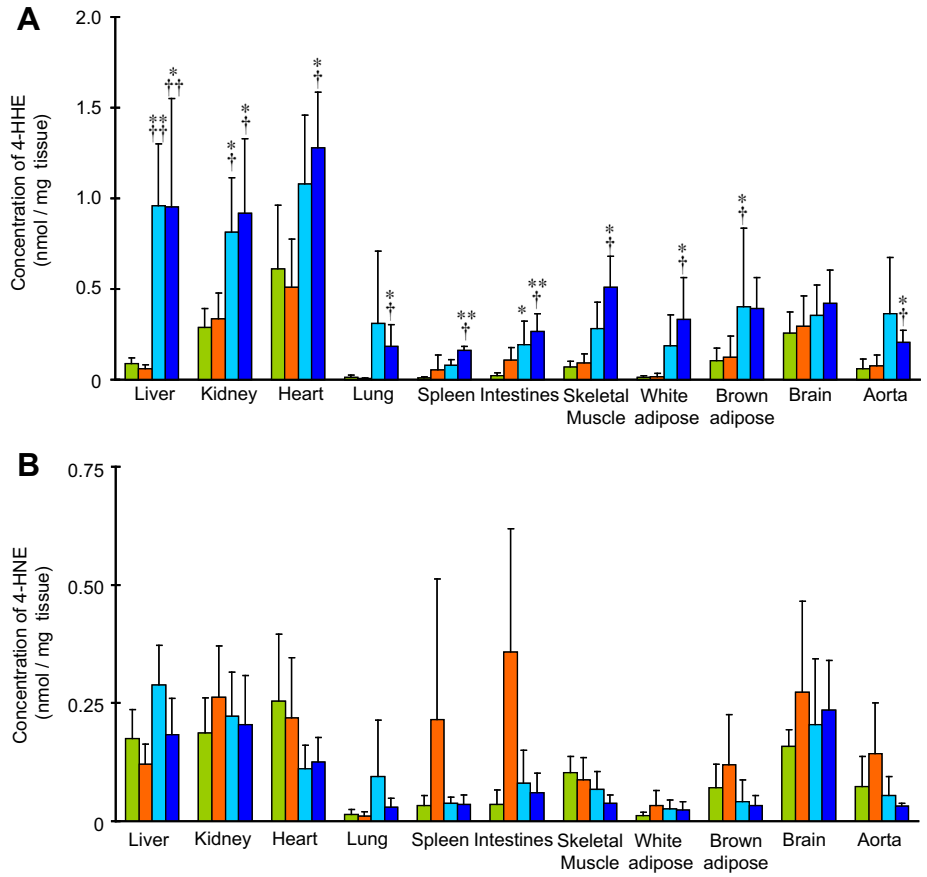

Fig. 3. Concentration of 4-HHE and 4-HNE in multiple organs in C57BL/6 mice. Green bar; control diet with 3 weeks, Orange bar; safflower oil diet with 3 weeks Light blue bar; fish oil diet with 1 week, Dark blue bar; fish oil diet with 3 weeks. (A) Concentration of 4-HHE. (B) Concentration of 4-HNE were measured using LC/MS/MS. Published data applicable to the aorta [11] are also included for comparison. Each value represents the mean ± SD of four animals. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control diet group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with safflower oil diet group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

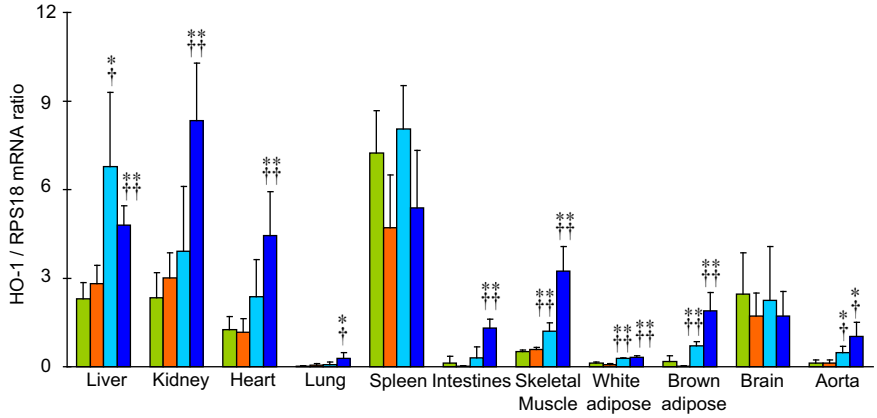

Fig. 4. HO-1 mRNA expression in multiple organs in C57BL/6 mice. Green bar; control diet with 3 weeks, Orange bar; safflower oil diet with 3 weeks Light blue bar; fish oil diet with 1 week, Dark blue bar; fish oil diet with 3 weeks. The relative mRNA expression of HO-1 was analyzed quantitatively using real-time RT-PCR. The results were normalized to RPS18 and expressed as fold increase over control. Each value represents the mean ± SD of four animals. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control diet group. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with safflower oil diet group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

present study and Awada et al. [35] (nM or fM in each tissue even after fish-oil treatment) may account for this discrepancy. HO-1 has been recognized as an important protective factor against various diseases because of its anti-oxidative, anti-inflammatory, anti-proliferative, and anti-apoptotic effects [36–38]. Therefore, increases in HO-1 expression in various tissues by fish-oil supplementation can be considered to be the explanation for the pleiotropic effects of n-3 PUFAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.085.

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