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Omega-3 polyunsaturated fatty acid has an anti-oxidant effect via the Nrf-2/HO-1 pathway in 3T3-L1 adipocytes

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ABSTRACT

Oxidative stress is produced in adipose tissue of obese subjects and has been associated with obesity-related disorders. Recent studies have shown that omega-3 polyunsaturated fatty acid (ω3-PUFA) has beneficial effects in preventing atherosclerotic diseases and insulin resistance in adipose tissue. However, the role of ω3-PUFA on adipocytes has not been elucidated. In this study, 3T3-L1 adipocytes were treated with ω3-PUFA and its metabolites, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or 4-hydroxy hexenal (4-HHE). ω3-PUFA and its metabolites dose-dependently increased mRNA and protein levels of the anti-oxidative enzyme, heme oxygenase-1 (HO-1); whereas no changes in the well-known anti-oxidant molecules, superoxide dismutase, catalase, and glutathione peroxidase, were observed. Knockdown of nuclear factor erythroid 2-related factor 2 (Nrf-2) significantly reduced EPA, DHA or 4-HHE-induced HO-1 mRNA and protein expression. Also, pretreatment with ω3-PUFA prevented H_2O_2-induced cytotoxicity in a HO-1 dependent manner. In conclusion, treatment with EPA and DHA induced HO-1 through the activation of Nrf-2 and prevented oxidative stress in 3T3-L1 adipocytes. This anti-oxidant defense may be of high therapeutic value for clinical conditions associated with systemic oxidative stress.

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1. Introduction

It is well established that obesity increases oxidative stress, which is predictive of vascular disease and associated complications, including vascular dysfunction, insulin resistance, and decreased levels of adiponectin [1–7]. Insulin resistance in adipose tissue leads to systemic insulin resistance in liver and muscle. Therefore, protecting adipocytes against oxidative stress could have clinical value in combating obesity-related metabolic diseases; however, the molecular mechanisms for how oxidative stress is ameliorated in adipocytes have not been fully elucidated.

There is considerable evidence suggesting that omega-3 polyunsaturated fatty acid (ω3-PUFA), and its metabolites, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have a wide range of biological effects, including anti-inflammatory effects, reduction of cytokine production and decreasing oxidative stress [8]. Recent studies have shown that ω3-PUFA has beneficial effects in the prevention of obesity and insulin resistance, and it has been proposed that ω3-PUFA stimulates muscle glycogen synthesis [9] and improves obesity-induced insulin resistance in the liver [10,11]. It has also been reported that the effects of ω3-PUFA are mediated by the peroxisome proliferator-activated receptors (PPAR)-α dependent pathway in diet-induced obese mice [9–13]. Furthermore, ω3-PUFA has been shown to modulate anti-oxidant enzyme activity, such as superoxide dismutase (SOD) and catalase in livers from diabetic rats fed a high fat diet [14]. There is therefore growing evidence to suggest that ω3-PUFA has an anti-oxidative stress effect, and that the subsequent effects on adipocytes could be of high therapeutic value for obesity-associated disorders. However, the direct mechanistic effect of ω3-PUFA on adipocytes is not yet fully understood.

In the present study, we demonstrate that ω3-PUFA induces the anti-oxidative enzyme, HO-1, through Nrf-2, and show that ω3-PUFA prevented H_2O_2-induced cytotoxicity in a HO-1 dependent manner. Our results indicate that ω3-PUFA exerts effects as an anti-oxidant molecule in adipocytes.

Abbreviations: ω3-PUFA, ω3-polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; 4-HHE, 4-hydroxy hexenal; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H quinone oxidoreductase 1; ROS, reactive oxygen species; NBT, nitro blue tetrazolium.

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2. Materials and methods

2.1. Reagents

Anti-HO-1, anti-Nrf-2, horseradish peroxidase-linked anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco's Modified Eagle's Medium (DMEM), and fetal calf serum (FCS) were obtained from Life Technologies (Grand Island, NY). BioMax MR film was obtained from Eastman Kodak Co. (Rochester, NY). EPA, DHA, 4-HHE, 18-hydroxy-5Z, 8Z, 11Z, 14Z, 16E-eicosapentaenoic acid (18-HEPE), 10(S),17(S)-DiHDoHE, and GW9508 were purchased from Cayman (Ann Arbor, MI). All other reagents and chemicals were from standard suppliers.

2.2. Cell cultures

3T3-L1 cells, provided by Dr. J.M. Olefsky (University of California, San Diego, CA), were cultured and differentiated into adipocytes, as previously described [15]. Prior to each experiment, the adipocytes were trypsinized and reseeded in the appropriate culture dishes [16].

2.3. Transfection study

The duplexes of siRNA, targeting HO-1, Nrf-2, G-protein coupled receptor (GPR) 120 mRNA, and a negative control were purchased from Santa Cruz Biotechnology. On day 5 post-differentiation, electroporation was performed using Amaxa Nucleofector technology (Amaxa, Cologne, Germany), as previously described [17].

2.4. Western blotting

Total protein samples were prepared, as previously described [17], and were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being transferred to membranes. Membranes were probed with antibodies against HO-1, and β-actin.

2.5. RNA preparation from adipocytes and quantitative RT-PCR

Total RNA samples were prepared and reverse transcription (RT)-PCR reactions were performed, as previously described [18]. Gene expression levels were calculated after normalization to the standard housekeeping genes, GAPDH, using the delta/delta Ct methods, as previously described [19], and expressed as relative mRNA levels when compared with the internal control. Primer sets were as follows: mouse HO-1, 5′-CGAAACAAGCAGAACCCAGTCT-3′ and 5′-AGCCCTTCGGTGCAGCT-3′; mouse catalase, 5′-ACAAGAAAGAAACCTGATGGAGAGA-3′ and 5′-GCCAGAAGAGAAACCCACAGA-3′; mouse MnSOD, 5′-CTTCAATAAGGAGCAAGGTCGC-3′ and 5′-CACACGTCAATCCCCAGC-3′; mouse CuZnSOD, 5′-AATGGTGGTCCATGAGAAACAAG-3′ and 5′-GCAATCCCAATCACTCCACA-3′; mouse Nqo1, 5′-GTCTTCTCTGAATGGGCCAG-3′ and 5′-TTTAGGGTCGTC TTGGCAAC-3′; mouse Gpx, 5′-GGGCAAGGTGCTGCTCATTG-3′ and 5′-AGAGCGGGTGAGCCTTCTCA-3′; mouse Nrf-2, 5′-GGTTCAGTGACTCGGAAATGG-3′ and 5′-GAGAATGTGCTGGCTGTGCT-3′ and mouse GPR120, 5′-ACCAAGTCAATCGCACCCACTTCCC-3′ and 5′-GGTCTCCACGACGCTCAACACCAAC-3′ and mouse GAPDH, 5′-GCCTGCTTCACCACCTTCT-3′ and 5′-ATGGCCTTCCGTGTTCCT-3′.

2.6. NBT assay

Reactive oxygen species (ROS) production was detected by nitroblue tetrazolium (NBT) assay [20]. 3T3-L1 adipocytes were incubated with 100 μM of BSA, EPA or DHA for 1 h pretreated with or without *N*-acetyl-L-cysteine (NAC). Cells were stimulated with or without H_2O_2 for 24 h and then incubated for 90 min in PBS containing 0.2% NBT. NBT is reduced by ROS to a dark-blue insoluble form of NBT called formazan. Formazan was dissolved in 50% acetic acid and the absorbance was determined at 560 nm.

2.7. Statistical analysis

All values are expressed as mean ± SEM unless otherwise stated. Scheffe's multiple comparison test was used to determine the significance of any differences between more than three groups. A p value less than 0.05 was considered significant.

3. Results

3.1. EPA and DHA induced anti-oxidative enzyme HO-1 and NQO1 in 3T3-L1 adipocytes

Since ω3-PUFA is considered an anti-oxidant molecule in aortic cells [21], we evaluated whether it also has an anti-oxidative effect on adipocytes. Unexpectedly, treatment with EPA and DHA in 3T3-L1 adipocytes had no effect on the expression of manganese superoxide dismutase (MnSOD), copper/zinc SOD (Cu/ZnSOD), catalase or glutathione peroxidase (GPx) mRNA expression (Fig. 1A–D). However, treatment with each reagent for 8 h increased the anti-oxidant enzyme, HO-1, and NAD(P)H quinone oxidoreductase 1 (NQO1) mRNA expression (Fig. 1E and F). Additionally, HO-1 mRNA expression by EPA and DHA was in a dose-dependent manner (Fig. 1G). We found that each reagent caused a maximal increase in HO-1 mRNA expression for 8 h (Fig. 1G). Furthermore, treatment with EPA and DHA for 24 h increased the intracellular protein expression of HO-1 (Fig. 1H).

3.2. 4-HHE induced HO-1 mRNA in 3T3-L1 adipocytes

ω3-PUFA is considered to be metabolized to its metabolites, and these metabolites exhibit beneficial effects. 18-HEPE is produced by non-enzymatic oxidation of EPA, and 10(S),17(S)-DiHDoHE, also referred to as protectin D1, is a DHA metabolite. As they have been reported to be representative members of DHA- and EPA-derived lipid mediators and mimic beneficial actions as potent protective, anti-inflammatory, and pro-resolution properties [10,11], we examined whether 18-HEPE and 10(S),17(S)-DiHDoHE increased HO-1 mRNA expression and found neither induced HO-1 in 3T3-L1 adipocytes (Fig. 2A).

We then examined the effect of another end-product aldehyde of ω3-PUFA peroxidation, 4-HHE. Interestingly, 4-HHE induced HO-1 mRNA expression (Fig. 2A). 4-HHE caused a maximal increase in HO-1 mRNA expression after 6 h (Fig. 2B). Treatment with 4-HHE dose-dependently increased HO-1 mRNA expression (Fig. 2C).

As it has been reported that DHA leads to an increase in glucose uptake via GPR120 in 3T3-L1 adipocytes [22], we stimulated GPR120 with synthetic agonists, GW9508, to examine whether GPR senses the anti-oxidant enzyme, HO-1. Our results showed that GW9508 did not induce HO-1 mRNA expression (Fig. 2D).

3.3. EPA, DHA and 4-HHE induced HO-1 expression through Nrf-2 in 3T3-L1 adipocytes

The findings presented in Fig. 2 suggest that ω3-PUFA induced HO-1 expression, not through the GPR120 pathway. To explore which pathways are critical for the induction of HO-1 by ω3-PUFA, we transfected 3T3-L1 adipocytes with siRNA against Nrf-2, a

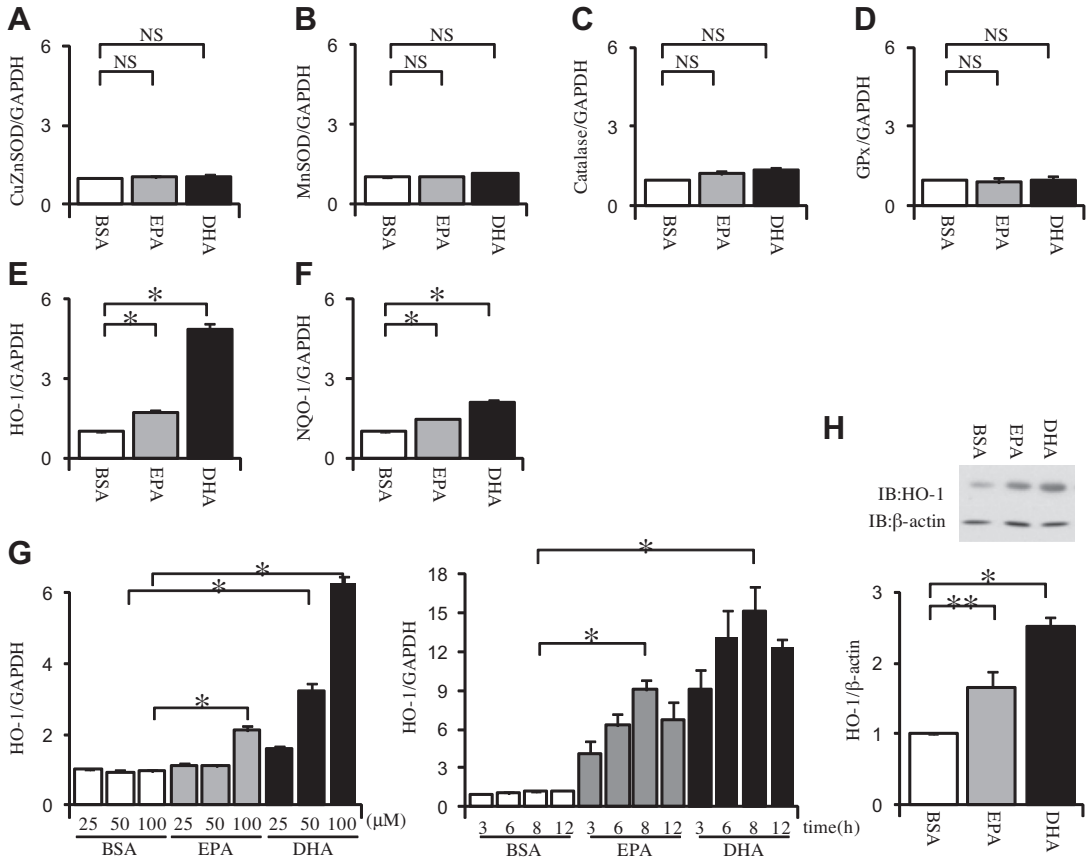

Fig. 1. Treatment with EPA and DHA induced the antioxidative enzyme HO-1 and NQO1 in 3T3-L1 adipocytes. 3T3-L1 adipocytes were pretreated with the concentration indicated (G) or 100 μM (A–G) BSA, EPA or DHA for the indicated time (G), or 8 h (A–G). Total RNA was purified and quantitative RT-PCR was performed. Data are presented as the relative expression and normalized to GAPDH. (H) 3T3-L1 adipocytes were stimulated with 100 μM BSA, EPA or DHA, and lysed and immunoblotting (IB) was performed with indicated antibodies. The graph show the mean ± S.E. and the values are expressed as fold increase compared to control. β-actin was showed as an endogenous control. *P < 0.01, **P < 0.05, compared with control. NS, no significant difference.

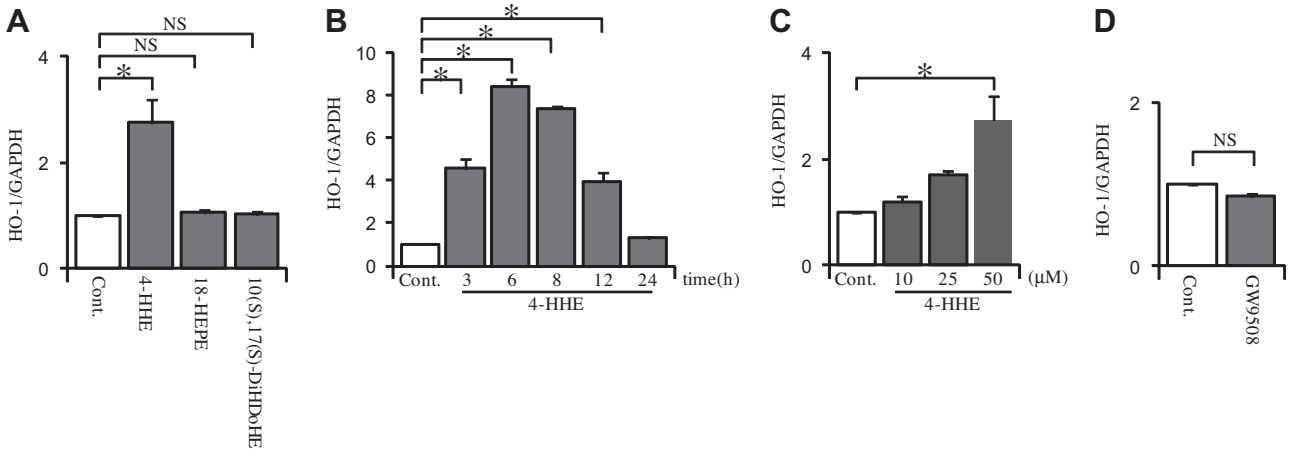

Fig. 2. 4-HHE induced HO-1 mRNA in 3T3-L1 adipocytes. The 3T3-L1 adipocytes were stimulated with concentration indicated (C) or 50 μM 4HHE, 50 nM 18-HEPE, 50 nM 10(S),17(S)-DiHDoHE, and 20 μM GW9508 for the indicated time (B) or 6 h (A, D). Total RNA was purified and quantitative RT-PCR was performed. Data are presented as the relative expression and normalized to GAPDH. Error bars represent the mean ± S.E. *P < 0.01, compared with control. NS, no significant difference.

well-known upstream regulator of HO-1, and GPR120, by electroporation. Twenty-four hours after electroporation, we observed a 70% and 60% decrease in both Nrf-2 and GPR120 mRNA in cells transfected with siRNA against Nrf-2 (Fig. 3A) or GPR120 (Fig. 3D), respectively, when compared with cells transfected with control siRNA. Depletion of Nrf-2 abolished EPA or DHA induced HO-1 expression (Fig. 3A and B). 4HHE also had no effect on HO-1 expression in Nrf-2 knock down cells (Fig. 3C). However, depletion of GPR120 had no effect on ω3-PUFA induced HO-1 expression (Fig. 3D). These findings indicate that ω3-PUFA increased the expression of HO-1 through the Nrf-2 pathway, but not through the GPR120 pathway.

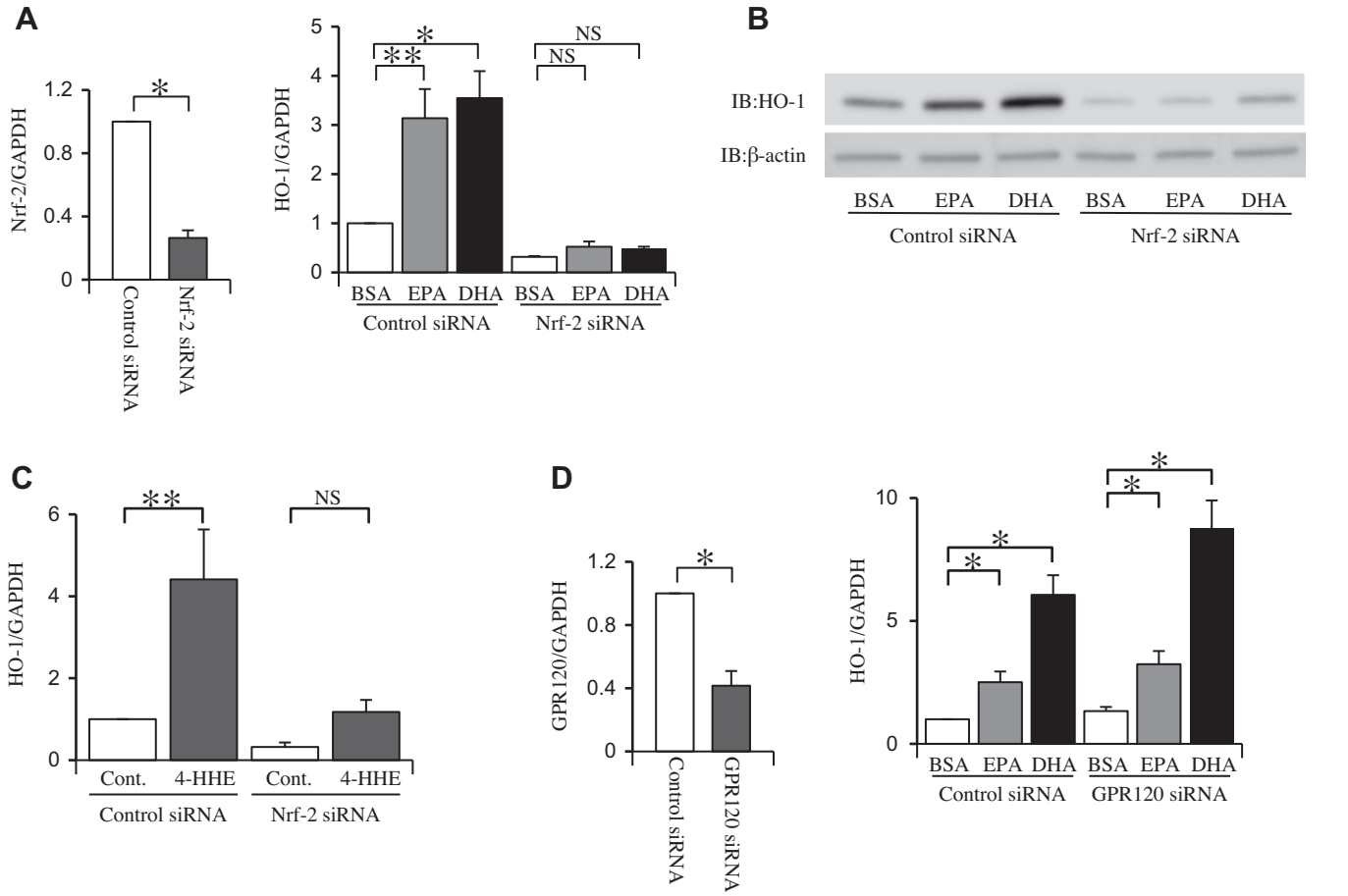

Fig. 3. EPA, DHA and 4-HHE induced HO-1 expression through Nrf-2 in 3T3-L1 adipocytes. (A–C) 3T3-L1 adipocytes were electroporated with siRNA against Nrf-2 or control siRNA for 24 h. After electroporation, treatment of 100 μM BSA, EPA, DHA (A, B), or 50 μM 4-HHE (C), and then HO-1 mRNA (A, C) or protein (B) was evaluated by quantitative RT-PCR or Western blotting. (D) 3T3-L1 adipocytes electroporated with siRNA against GPR120 or control siRNA, were stimulated with 100 μM BSA, EPA or DHA. Total RNA was extracted and the relative mRNA expression of HO-1 and GPR120 was analyzed by RT-PCR. Results were normalized to GAPDH and expressed as fold increase over BSA-treated control siRNA electroporated cells. Values are expressed as the means ± S.E. $^{*}P < 0.01$, $^{**}P < 0.05$, compared with control. N.S., no significant difference.

3.4. EPA and DHA protects against H_2O_2-induced ROS production in 3T3-L1 adipocytes

To evaluate the physiological role of ω3-PUFA induced HO-1 expression, we assessed the cytoprotective effects of EPA and DHA against H_2O_2-induced oxidative stress. We examined cell viability, assessed using a NBT assay, after the induction of oxidative stress by H_2O_2 for 24 h and found that EPA and DHA partially prevented H_2O_2-induced ROS production, compared with BSA alone (Fig. 4A). Administration of NAC attenuated H_2O_2-induced NBT reduction, but EPA and DHA had no additive effect, suggesting that ω3-PUFA exerts effects as an anti-oxidant molecule and prevented H_2O_2-induced ROS production.

To further confirm the role of HO-1 on ω3-PUFA's protective effect from H_2O_2-induced ROS production, we performed a NBT assay in HO-1 knockdown cells. Transfection with HO-1 siRNA reduced the expression of HO-1 mRNA by close to 80% (Fig. 4B). As shown in Fig. 4C, attenuation of ROS production by EPA and DHA was significantly suppressed in these cells, suggesting that ω3-PUFA attenuates ROS production through HO-1 induction.

4. Discussion

In this study, we show a novel direct anti-oxidant role of ω3-PUFA on adipocytes. ω3-PUFA is able to increase HO-1 expression through the activation of Nrf-2 in 3T3-L1 adipocytes. We also reveal a preventive effect of ω3-PUFA against H_2O_2-induced ROS production.

Chronic low-grade adipose tissue inflammation is a major cause of systemic insulin resistance that exists in obesity-related disorders such as type 2 diabetes. Although the roles of tissue macrophages and adipocytes in this process have been well documented, recent studies have focused on the direct effect of ω3-PUFA within a macrophage. For example, one study has reported that a biological mechanism responsible for the anti-inflammatory effects of ω3-PUFA is by way of GPR120 in macrophages [22]; and in another, it was reported that EPA increases adiponectin levels and improves insulin sensitivity through the inhibition of inflammatory responses in macrophage *in vivo* [23]. These reports suggest that ω3-PUFA affects adipose tissue macrophages. However, the direct effect of ω3-PUFA on the adipocytes themselves has not been elucidated. In this regard, in 3T3-L1 adipocytes, we show, for the first time, that ω3-PUFA leads to an increase in expression of HO-1 via the Nrf-2 pathway and inhibits H_2O_2-induced ROS generation. Results demonstrate that ω3-PUFA exerts effects as an anti-oxidant and cytoprotective component within adipocytes.

To elucidate the molecular mechanism producing the anti-oxidant role of ω3-PUFA on adipocytes, we examined anti-oxidant enzymes. We showed that treatment with EPA and DHA in 3T3-L1 adipocytes increased HO-1 and NQO1 mRNA expression; however,

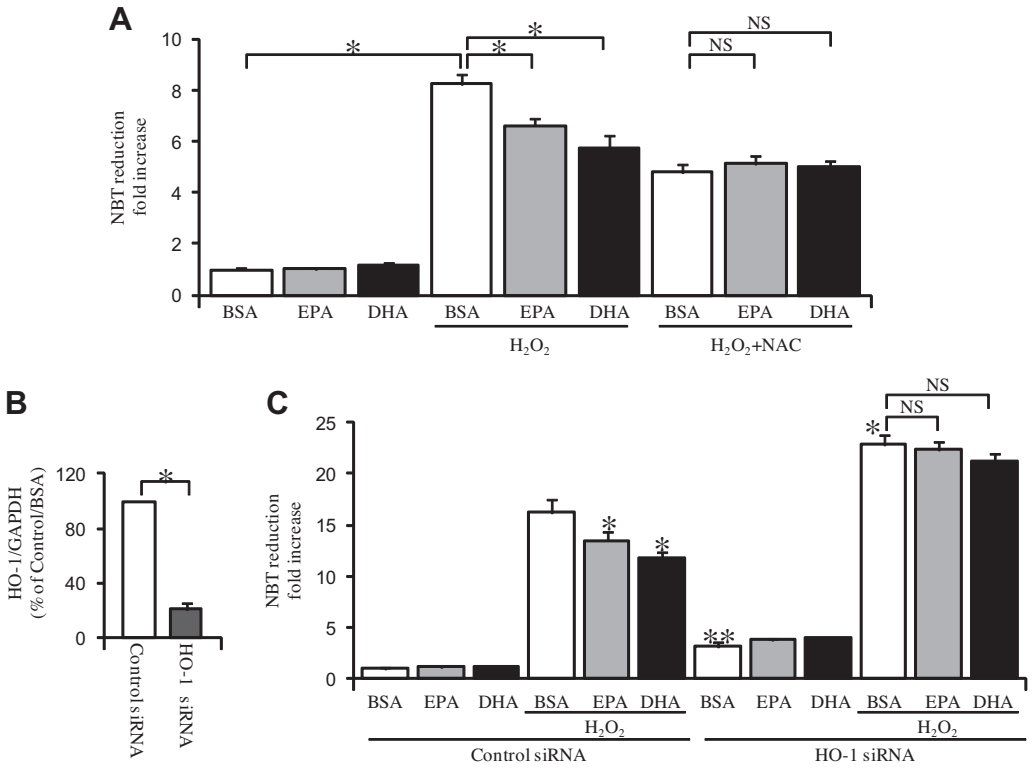

Fig. 4. Preventive effects of EPA and DHA on H_2O_2-induced cytotoxicity in 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes pretreated with or without 10 mM NAC for 1 h, were treated with 100 μM BSA, EPA, or DHA for 1 h, and then stimulated with 500 μM H_2O_2 for 24 h. (B) 3T3-L1 adipocytes were electroporated with control siRNA or HO-1 siRNA. Cells pretreated with 100 μM BSA, EPA, or DHA were stimulated with 500 μM H_2O_2 for 24 h. Total RNA was purified and quantitative RT-PCR performed. Data are presented at the relative expression and normalized to GAPDH. ROS production was measured by NBT assay as described Section 2. Values represent the means ± S.E. of four experiments, compared with BSA-treated H_2O_2-untreated HO-1 knockdown cells. $^{*}P < 0.01$, compared with BSA treated H_2O_2-stimulated control cells. $^{**}P < 0.01$, compared with BSA treated control cells. N.S., no significant difference.

they had no effect on the expression of MnSOD, Cu/ZnSOD, catalase and GPx mRNA expression. ω3-PUFA is known as one of the PPARs activators [24], and PPARs ligands may activate anti-oxidant enzymes such as SOD and catalase in the liver and skeletal muscle [25–27]. Our results indicate that ω3-PUFA may have an anti-oxidant role through a distinct mechanism in adipocytes.

We also observed that there were differences in the impact of EPA and DHA on the anti-oxidative effects. DHA significantly increased HO-1 mRNA and protein levels more than EPA, and it also had a protective effect from H_2O_2-induced ROS production. In the context of anti-inflammation, it has been reported that DHA may also be more effective than EPA in alleviating LPS-induced pro-inflammatory cytokine production in macrophages [28], although there is evidence to suggest that ω3-PUFA can mediate the activity of transcription factors, such as NF-κB [29,30]. We considered that the differences observed between EPA and DHA might result from differences in the metabolism of each reagent. 4-HHE, which is metabolized from ω3-PUFA, was able to increase HO-1 expression through Nrf-2 activation earlier than from EPA and DHA (6 vs. 8 h). The time needed to metabolize to 4-HHE from EPA or DHA might cause this time lag. Also, we found that DHA, more so than EPA, causes an increase in intracellular 4-HHE (unpublished data). Further investigation into how EPA or DHA metabolizes to 4-HHE, and how 4-HHE activates Nrf-2, may solve this issue.

Although ω3-PUFA has been reported to increase glucose uptake via GPR120 in 3T3-L1 adipocytes [22], in the current study, we observed that EPA and DHA had an anti-oxidant effect through the Nrf-2/HO-1 pathway. This observation, together with a recent study showing that 4-HHE, a peroxidation product of ω3-PUFA, is able to stimulate HO-1 expression through the activation of Nrf-2 and has a preventive effect against oxidative stress-induced cytotoxicity in vascular endothelial cells [31], leads us to believe that our data demonstrate that ω3-PUFA exerts effects as an anti-oxidant and cytoprotective component in adipocytes. Furthermore, depletion of HO-1 increased basal (lane 1 vs. lane 7) and H_2O_2-induced (lane 4 vs. lane 10) ROS production (Fig. 4C), suggesting that HO-1 is involved with the anti-oxidant effects seen in adipocytes.

Although the role of the Nrf-2/HO-1 pathway is still being considered, HO-1 is a well-known molecular response to oxidative stress, and it is becoming increasingly clear that appropriate low levels of oxidative stress protect against upcoming severe oxidative stress. This is an area of current interest, called preconditioning. In this regard, ω3-PUFA increased HO-1 protein expression by 1.5- to 2.5-fold (Fig. 1H) and protected against H_2O_2-induced ROS production (Fig. 4A). The levels of HO-1 induction observed in our study might be an appropriate level of expression to prevent oxidative stress. In ischemic heart disease, preconditioning has been well established. However, the potential of preconditioning procedures to oxidative stress in adipocytes requires investigation.

It has been suggested that fish, or fish-oil, as a dietary supplement may reduce the risk of cardiovascular disease, lower blood pressure, and modify lipid metabolism and inflammation [32,33]. These beneficial effects might be through HO-induction and anti-oxidant effects in adipocytes. Further *in vivo* study is needed, but it is of interest that there is an improvement shown in adipocytes to the effects of oxidative stress by dietary supplementation of ω3-PUFA. It is also of interest that the reduction of oxidative stress attenuates obesity-related systemic insulin resistance.

In summary, we show, for the first time, that ω3-PUFA has an anti-oxidative stress effect through expression of HO-1 via the Nrf-2 pathway in 3T3-L1 adipocytes. In a broader context, our findings strongly suggest that an enhancement of the anti-oxidant

defense has high therapeutic value, and HO-1 should be one of the therapeutic approaches because of its dynamically modulatory properties.

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