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Docosahexaenoic acid improves vascular function via up-regulation of SIRT1 expression in endothelial cells

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ABSTRACT

n-3-Polyunsaturated fatty acids (PUFAs) protect against myocardial infarction, arteriosclerosis and high blood pressure by stimulating endothelial nitric oxide synthase (eNOS) to increase nitric oxide (NO) production. However, the mechanism remains to be elucidated. This study investigated the role of SIRT1 in the protective effects of docosahexaenoic acid (DHA) in vascular endothelial cells. Exposure of human umbilical vein endothelial cells (HUVECs) to 0.3–30 μM DHA did not affect cell viability, and DHA treatment dose-dependently increased SIRT1 expression. The DHA-mediated increase in SIRT1 expression induced eNOS deacetylation, increasing endothelial NO. However, inhibition of SIRT1 inhibited DHA-mediated increases in NO production. This effect was mediated via deacetylation of lysines 496 and 506 in the eNOS calmodulin-binding domain. The effects of DHA were also demonstrated in rat aortic rings, in which DHA treatment increased SIRT1 expression and bioavailable NO. Our results demonstrate that SIRT1 plays an important role in DHA-mediated increases in bioavailable NO via decreased eNOS acetylation.

1. Introduction

The health benefits of n-3-polyunsaturated fatty acids (PUFAs) were first recognized in a study of Greenland Eskimos, who had an extremely high intake of PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), with a low risk of cardiovascular disease [1]. PUFAs are beneficial to the regulation of many physiological functions, including vascular endothelium function, the immune response, blood pressure regulation, lipid status, cell proliferation, blood clotting, and inflammation [2–4]. DHA enhances vascular endothelial function and consequently decreases arterial blood pressure and fatal arrhythmia in rats and humans [3]. Moreover, DHA enhances vasodilator mechanisms and attenuates constrictor responses via endothelial nitric oxide synthase (eNOS) activation in forearm microcirculation [3] and coronary artery endothelial cells [5]. Among the many vasoactive mediators modulating vascular function, nitric oxide (NO) is the most important endothelium-dependent vasodilating mediator [6–8]. Many stimulators, including shear stress and DHA, regulate NO production through the phosphorylation of eNOS [5,9] at ser1177 and thr495 [10,11].

Sir2 (silent information regulator 2) is a class III NAD-dependent histone deacetylase (HDAC) [12]. SIRT1, the mammalian ortholog of Sir2, targets deacetylation of lysine residues in histone and non-histone targets such as eNOS and promotes endothelium-dependent vasodilation via eNOS deacetylation [13]. In addition to modulating deacetylase activity in proteins, SIRT1 can also indirectly modulate AMP-activated protein kinase (AMPK) via the upstream regulator LKB1 (serine-threonine liver kinase B1) [14]. AMPK activates NOS by phosphorylation of eNOS at ser633 [15,16]. Meanwhile, some studies reported that AMPK can also function as a SIRT1 activator [14]. SIRT1-LKB1-AMPK signaling is a crucial mediator of a variety of metabolic functions and the aging process, including endothelium-dependent vasodilation [14,17,18]. Among dietary supplements, fish oil consumption has demonstrated evidence of a protective effect against several metabolic syndromes, such as diabetes and cardiovascular disease [3,4,19].

A recent study reported that dietary supplementation with DHA was effective in reversing the reduction of SIRT1 levels in rats with mild traumatic brain injury [20]. However, in endothelial cells, the exact mechanisms of eNOS activation and augmented NO bioavail-

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ability have not been fully elucidated. To address the hypothesis that the effects of DHA on eNOS function are mediated in part by SIRT1, we investigated the role of SIRT1 in DHA-induced eNOS regulation and endothelium-dependent vasodilation.

2. Materials and methods

2.1. Cell culture and cell viability assay

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and cultured in an endothelial growth medium. Sub-confluent, proliferating HUVECs at passages 2–8 were used. The effects of DHA on HUVEC viability were measured using an ADAM-MC automatic cell counter (Digital Bio, Seoul, South Korea) that functions by assessing propidium iodide (PI) staining [20].

2.2. Antibodies and immunoblotting

Anti-eNOS and anti-SIRT1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-acetyl lysine antibodies from Cell Signaling Technology (Beverly, MA, USA). Lysine acetylation of eNOS was detected by immunoblotting of immunoprecipitated eNOS with an acetyl lysine (Ac-K) antibody. Immunoprecipitations of eNOS were performed by incubating 2 μg of antibody with 1 mg of cell lysate or tissue homogenate overnight, followed by addition of a 30-μL protein A-Sepharose slurry (Amersham, Parsippany, NJ, USA) for 4 h. After washing, the immunoprecipitates were boiled in SDS–PAGE gel loading buffer, separated by electrophoresis, transferred to a nitrocellulose filter, and probed with eNOS and Ac-K antibodies and the appropriate peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA). Chemiluminescent signal was developed using Super Signal West Pico or Femto Substrate (Pierce, Rockford, IL, USA). Western blotting of 50 μg of whole-cell lysates or tissue homogenate was similarly performed using appropriate primary and secondary antibodies. Blots were imaged and band densities quantified with a Gel Doc 2000 Chemi Doc system using the Quantity One software (Bio-Rad, Hercules, CA). Values were normalized to a β-actin loading control.

2.3. Real-time polymerase chain reaction

Total RNA from cells or the thoracic aorta of the mice was isolated using the acid guanidinium thiocyanate–phenol–chloroform method. Real-time polymerase chain reaction (PCR) was performed using the Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the Super Script III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA). Primers for human SIRT1 were as follows: 5-AAGTACAATCCACTCC GGAATGA-3 and 5-GGGCCCCAGGGATGAAG-3. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers for human GAPDH were as follows: 5-ATGA CATCAAGAAGGTGGTG-3′ and 5-CATACCAGG AAAATGAGCTTG-3. Dissociation curves were monitored to check the aberrant formation of primer-dimers.

2.4. RNA interference and transfection

Validated stealth small interfering (siRNA) for SIRT1 (5-GCAACAGCAUCUUGCCUGAUUUGUA-3, nucleotides 1152–1175 of human SIRT1 mRNA) and the appropriate control RNAi were purchased from Invitrogen (Leiden, Netherlands). Single transfection of 10–100 nM siRNA duplexes was performed using the Lipofectamine 2000 reagent (Invitrogen). The cells were incubated at 37 °C in a CO_2 incubator for gene knockdown.

2.5. Histological analysis

De-paraffinized rat and mouse aortic ring sections were permeabilized and processed using the Vectastain Universal Quick Kit (PK-8800, Vector Laboratories). A primary antibody against SIRT1 was used at a 1:50 dilution, followed by incubation with a biotinylated secondary antibody, and visualization using a streptavidin peroxidase solution, 3,3-diamino benzidine peroxidase substrate, and hematoxylin counterstain. Sections were digitally imaged using a Zeiss Axiovert 200 microscope.

2.6. Nitrite and nitrate measurements

NO metabolites nitrite (NO_2^-) and nitrate (NO_3^-), the stable breakdown products of NO, were quantified using a commercially available kit (Nitrate/nitrite Fluorometric Assay Kit, Cayman Chemicals, Lexington, KY, USA), as per the manufacturer's instructions. The medium was deproteinized using a 10-kDa cutoff filter (Microcon YM10 Millipore). After subtraction of background fluorescence, values were normalized to obtain the total protein amount.

2.7. Vascular reactivity

Wistar-Kyoto rats (3–4 months old) were sacrificed via sodium pentobarbital overdose. A mid-sternal split was quickly performed, and the descending thoracic aorta excised carefully and placed in ice-cold Krebs buffer (118.3 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM KH_2PO_4/25 mM $NaHCO_3$/1.2 mM $MgSO_4$/11 mM glucose/0.0026 mM $CaNa_2$ EDTA). The aorta was cleaned of excess fat; cut transversely into 5–10 rings (2.0–3.0 mm), each of which was treated with 30 μM DHA and incubated at 37 °C for 24 h. Bioavailable NO was measured in aortic rings as described previously [13].

3. Results

3.1. DHA treatment has no effect on HUVEC viability

To investigate the impact of DHA on endothelial cell cytotoxicity, we examined the effect of DHA on HUVEC viability using PI staining. HUVEC viability was not affected by exposure to 0.3–30 μM DHA compared with non-treated cells (Supplementary Fig. 1 and 1B), indicating that DHA did not decrease HUVEC viability through nonspecific cytotoxicity.

3.2. DHA increases the SIRT1 expression level in HUVEC

To examine the relationship between DHA and SIRT1 expression, we first determined whether DHA affects SIRT1 protein expression. HUVECs were incubated with 0.3–30 μM DHA for 24 h or 10 μM DHA for approximately 3–24 h. Cells were then harvested and processed for western blot analysis and real-time PCR. SIRT1 protein expression was stimulated in a concentration-dependent manner by DHA (Fig. 1A). In addition, SIRT1 protein (Fig. 1B) and mRNA (Fig. 1C) expression was stimulated by DHA in a time-dependent manner.

3.3. DHA decreases eNOS acetylation and increases NO production in HUVECs

SIRT1 regulates a number of histone and non-histone proteins, including eNOS [13]. To investigate whether DHA deacetylates NOS in endothelial cells, we determined the acetylation of eNOS

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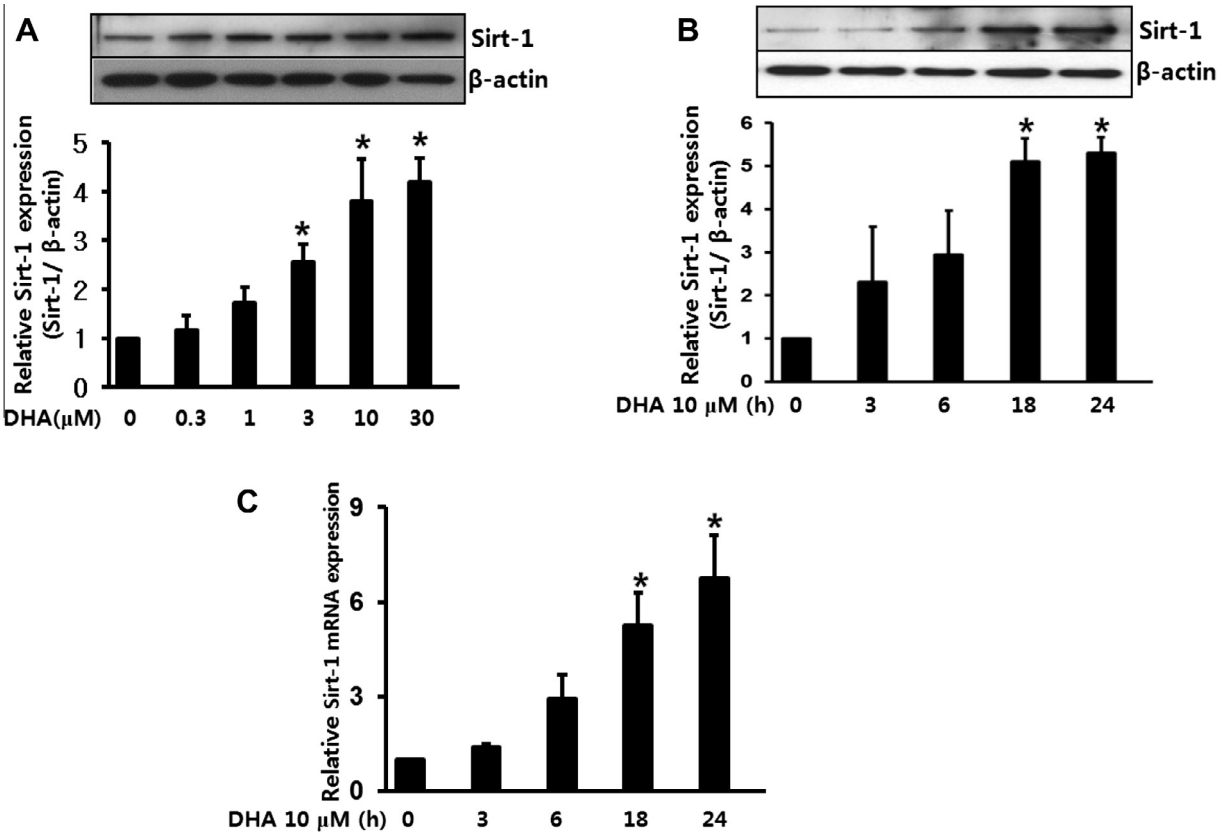

Fig. 1. Decosahexaenoic acid (DHA) increases endothelial SIRT1 expression. (A) DHA dose-dependently increased SIRT1 protein expression in human umbilical vein endothelial cells (HUVECs) treated with 0.3–30 μM DHA for 24 h. (B) DHA time-dependently increased SIRT1 protein expression in HUVECs treated with 10 μM DHA for 3, 6, 18, and 24 h. The cells were harvested and subjected to western blot analysis of SIRT1 (upper panels of A and B). β-Actin is shown as a loading control. SIRT1 expression levels were quantified by densitometric analysis (lower panels of A and B). All western blots are representative of three independent experiments. $^{*}P < 0.05$, compared with untreated cells. (C) DHA time-dependently increased SIRT1 RNA expression levels in HUVECs treated with 10 μM DHA for 3, 6, 18, and 24 h. Each bar represents the mean ± SE ($n = 3$). $^{*}P < 0.05$ compared with the control.

in HUVECs in which endogenous SIRT1 expression was knocked down with RNAi. The DHA-induced increase in SIRT1 expression level dose-dependently decreased the acetylation of endogenous eNOS on lysine residues (Fig. 2A). Similarly, inhibition of endogenous SIRT1 activity with nicotinamide increased the acetyl lysine-eNOS expression level (Fig. 2A). Furthermore, we investigated whether eNOS acetylation is altered during conditions that change the SIRT1 expression. The decrease in SIRT1 expression level by SIRT1 RNAi inhibited a reduction in eNOS acetylation on lysine residues by DHA (Fig. 2B). These results suggest that DHA decreases NOS acetylation in lysine residues via an increase in SIRT1 expression.

To determine whether DHA regulates eNOS-derived NO production, we performed nitrite and nitrate assays on media of HUVECs treated with DHA for 24 h. The SIRT1 expression level increased via a DHA-induced increase in eNOS-derived NO production (Fig. 3A) in parallel with DHA-induced increases in SIRT expression. However, DHA did not increase NO levels in HUVECs transfected with SIRT1 RNAi (Fig. 3A). Thus, DHA deacetylates NOS in HUVECs and eNOS acetylation in endothelial cells inversely correlates with DHA-induced increases in SIRT1 expression. To determine which lysine residues are modulated by SIRT1, we mutated lysines 496 and 506 in the eNOS calmodulin-binding domain (CBD) to non-acetylatable arginine (K496R and K506R). NO production in HEK293 cells transfected with eNOS (K496R) or eNOS (K506R) was significantly increased compared with the WT eNOS (Fig. 3B). Moreover, in contrast to WT eNOS, DHA did not increase NO production in HEK293 cells transfected with eNOS (K496R) or eNOS (K506R) (Fig. 3B). These results suggest that K496 and K506 in wild-type eNOS play important roles in the stimulation of NO production by DHA.

3.4. DHA regulates endothelium-dependent vascular tone

The role of DHA in associated endothelial function has been established [21]. Therefore, to determine whether SIRT1 expression plays an important role in DHA-mediated regulation of endothelium-dependent vascular tone, the vasomotor function of rat aortic rings was quantified after DHA treatment for 24 h. As shown in Fig. 4A and B, SIRT1 expression was increased in the DHA-treated endothelium compared with the control rings. Furthermore, compared with the control rings, rings treated with DHA exhibited no change in endothelium-dependent vasorelaxation (Fig. 4C). In contrast, the bioavailable NO level was increased markedly in the rings treated with DHA compared with the control rings (Fig. 4D). Thus, DHA plays an important role in the vascular bioavailability of NO by regulating SIRT1 expression.

4. Discussion

The class III HDAC SIRT1 is an NAD-dependent HDAC that acts as a master regulator of the stress response and energy homeostasis. SIRT1 expression lowers blood pressure, inflammation status, and insulin resistance [22]. Dietary supplements activating SIRT1 expression include DHA, polyphenols, and resveratrol [23]. We found that administration of DHA enhanced SIRT1 expression

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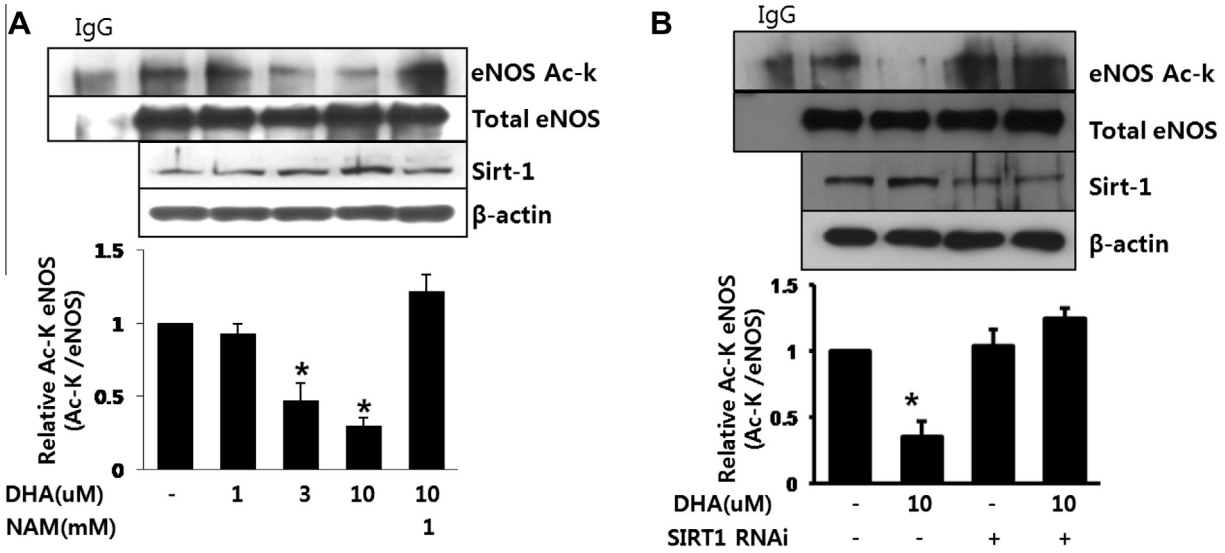

Fig. 2. Decosahexaenoic acid (DHA) deacetylates endothelial nitric oxide synthase (eNOS) in endothelial cells. (A) eNOS lysine acetylation in the presence of the SIRT1 inhibitor nicotinamide. The human umbilical vein endothelial cells (HUVECs) were treated with 1–10 μM DHA and 30 mM nicotinamide. Control cells were left untreated. (B) Effect of RNAi-mediated endogenous SIRT1 knockdown on eNOS lysine acetylation in cells treated with 10 μM DHA. Immunoprecipitates of eNOS from whole-cell lysates were immunoblotted with anti-eNOS and anti-acetyl lysine antibodies (upper panels of A and B). Non-immune immunoglobulin G in immunoprecipitates was used as a control. eNOS acetyl lysine levels were quantified by densitometric analysis (lower panels of A and B). All western blots are representative of three independent experiments. $^{*}P < 0.05$ compared with untreated cells.

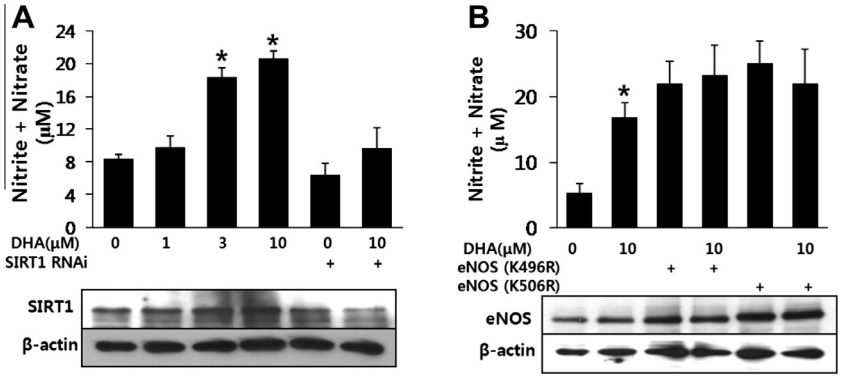

Fig. 3. Decosahexaenoic acid (DHA) increased endothelial nitric oxide (NO) bioavailability. (A) Metabolites of NO (nitrite and nitrate) were measured in the media of human umbilical vein endothelial cells (HUVECs) transfected with SIRT1 RNAi or control RNAi and treated with DHA. Control cells were left untreated. $^{*}P < 0.05$ compared with the control RNAi. (B) Nitrite and nitrate were measured in the media of HEK293-expressing wild-type (WT) eNOS, eNOS (K496R), or eNOS (K506R), or treated with DHA. $^{*}P < 0.05$ compared with WT eNOS.

and eNOS deacetylation, consequently increasing NO production and bioavailability. Inhibition of SIRT1 expression with nicotinamide or SIRT1 RNAi did not stimulate eNOS deacetylation or NO production by DHA, even though the same DHA dose was administered. Thus, the importance of SIRT1 expression in DHA-induced eNOS activation was investigated in this study.

The endothelium is pivotal in the regulation of vascular tone, which depends largely on eNOS-derived NO bioavailability. Endothelial dysfunction is associated with a decrease in endothelial NO bioavailability. Decreases in NO bioavailability reduce eNOS expression, decrease eNOS activity, and enhance eNOS degradation by reactive oxygen species (ROS) [24]. In addition to vasodilation, NO has anti-inflammatory and anti-thrombotic effects on the vascular wall.

In this study, a wide range of DHA treatments did not affect HUVEC viability. No recommended dose of omega-3 fatty acids for humans has been established; nevertheless, ingestion of fish at least twice per week in healthy individuals or 1 g/d of total EPA and DHA in patients with coronary artery disease is recommended for the prevention of fatal arrhythmia [25]. To reduce cardiovascular risk, higher doses of EPA and DHA (>3 g/d) are necessary [4]. Further investigations of dose-dependent safety in humans should be conducted.

Previous studies have shown that high doses of PUFAs decreased eNOS activity [26] and induced cell death [27]. In this study, SIRT1 protein expression was stimulated in a concentration-dependent manner by DHA in the HUVECs. In addition, when cells were incubated with 10 μM DHA for 3–24 h, SIRT1 protein and mRNA expression were time-dependently stimulated by DHA. Our results suggest that DHA benefits endothelial cell function at doses ranging from 0.3 to 30 μM of DHA.

Although it has been well established that DHA stimulates eNOS activity by phosphorylation at the ser1177 [26], the mechanism of action remains to be elucidated. We determined whether DHA modulated eNOS activity via SIRT1 expression in endothelial cells because SIRT1 regulates a number of histone and non-histone proteins, including eNOS. Some studies have suggested that DHA treatment results in increased acetylation and decreased deacety-

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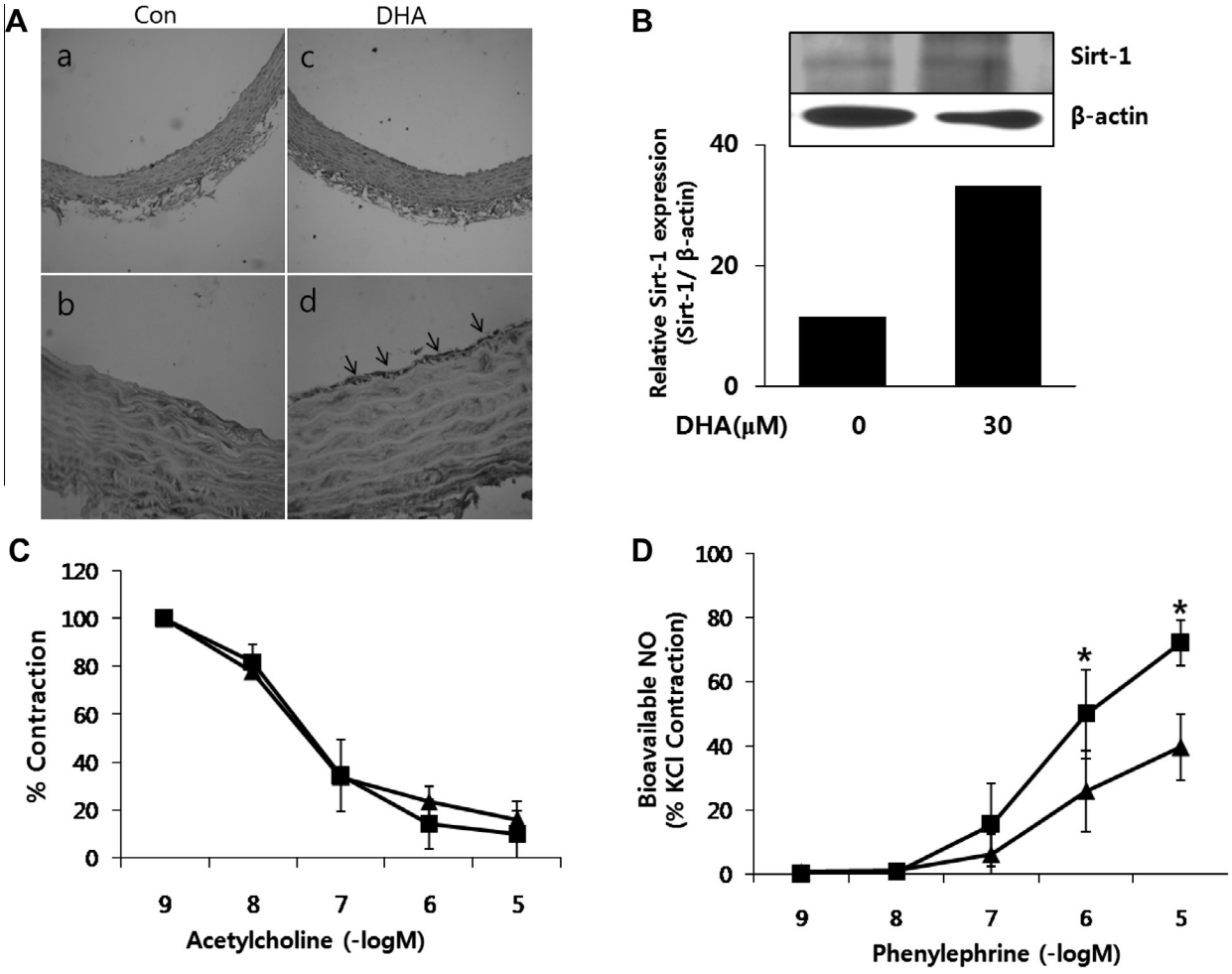

Fig. 4. Decosahexaenoic acid (DHA) increased SIRT1 expression and bioavailable nitric oxide (NO) in aortic rings. (A) Endothelial SIRT1 expression *ex vivo*. Rat aortic sections were treated with DHA (c and d) and stained for SIRT1. Original magnifications: ×100 (a and c) and ×400 (b and d). The arrows indicate SIRT1-positive staining of endothelial cells. (B) DHA increased SIRT1 protein expression in rat aortic rings treated with 10 μM DHA for 3, 6, 18, and 24 h. The rings were harvested and subjected to western blot analysis of SIRT1 (upper panel). β-Actin is shown as a loading control. SIRT1 expression levels were quantified by densitometric analysis (lower panel). The western blots are representative of three independent experiments. (C) Endothelium-dependent vasorelaxation ($n = 5$) and (D) bioavailable NO ($n = 5$) in rat aortic rings treated with DHA (■) and controls (▲). $^{*}P < 0.05$ compared with the control.

lation of H3 via down-regulated expression of the class I HDACs, HDAC1, HDAC2, and HDAC3 [28]. Our data indicate that DHA stimulates SIRT1-induced deacetylation of eNOS, suggesting that DHA may have a distinct effect on SIRT1 expression.

Two lysine residues in the CBD of bovine eNOS, which correspond to lysines 494 and 504 in human eNOS, were targeted in our studies. The CBD was chosen because Mattagajasingh et al. [13] reported that these residues are targeted by SIRT1. Moreover, addition of acetyl moieties to lysine residues in the CBD would cause similar steric and/or charge effects impacting eNOS activity as the addition of a phosphate group to photo-acceptor residues in the CBD. Therefore, we used non-acetylatable arginine (K496R and K506R) to determine that SIRT1 expression decreased eNOS metabolism via acetylation. eNOS deacetylation at lysines 496 and 506 in the bovine sequence increased eNOS activity and augmented NO production, while DHA significantly decreased lysine acetylated eNOS. We also demonstrated that DHA enhanced eNOS activity via increased SIRT1 expression. siRNA-mediated SIRT1 knock down did not result in a DHA-mediated decrease in eNOS acetylation and NO production. NO metabolite levels increased dose dependently with DHA treatment but did not stimulate NO production following siRNA-mediated SIRT1 knock down. Our results show that SIRT1 expression plays an important role in eNOS modulation in endothelial cells via deacetylation of K496 and K506 residues by DHA.

Furthermore, we examined the role of SIRT1 expression in endothelium-dependent vasodilation by DHA. We treated rat aortic rings with DHA for 24 h and observed higher overall SIRT1 expression in aortic rings treated with DHA than controls. However, it was interesting that the degree of vasodilation was not different between the DHA group and controls. Although DHA treatment was performed, endothelium-dependent vasodilation in response to acetylcholine was not improved (Fig. 4C), probably because the aortic rings were from normal, not atherosclerotic or hypertensive rats.

Our results demonstrate that increased SIRT1 expression activates eNOS to increase NO bioavailability. In DHA-treated rings, NO bioavailability increased markedly compared to control rings and in the non-hypertensive, healthy rats, DHA treatment markedly enhanced NO bioavailability. We predict that NO bioavailability would be greater in obese or hypertensive rats. Further studies of CR (caloric restriction), ROS, DHA, and SIRT1 have been conducted with the aim of extending the human lifespan by improving metabolism. In addition, further studies correlating the effects of DHA with longevity factors, such as SIRT1, are expected to reveal the "holy grail" for longevity research.

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In conclusion, our study demonstrated that DHA stimulates eNOS deacetylation by increasing SIRT1 expression level in endothelial cells. In addition, a DHA-induced increase in SIRT1 expression stimulated eNOS deacetylation and NO production. Therefore, NO bioavailability is increased by DHA, suggesting an important role for SIRT1 expression in endothelium-dependent eNOS-mediated vascular homeostasis.

Acknowledgments

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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.2013.06.049.

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