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Docosahexaenoic acid suppresses the expression of FoxO and its target genes☆

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Abstract

Docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid, has previously been shown to ameliorate obesity-associated metabolic syndrome. To decipher the mechanism responsible for the beneficial effects of DHA on energy/glucose homeostasis and the metabolic syndrome, 30 weaned cross-bred pigs were randomly assigned to three groups and fed *ad libitum* with a standard diet supplemented with 2% of beef tallow, soybean oil or DHA oil for 30 days, and the gene expression profile of various tissues was evaluated by quantitative real-time polymerase chain reaction. The DHA-supplemented diets reduced the expression of forkhead box O transcription factor (FoxO) 1 and FoxO3 in the liver and adipose tissue. DHA treatments also decreased the expression of FoxO1 and FoxO3 in human hepatoma cells, SK-HEP-1 and human and porcine primary adipocytes. In addition, DHA also down-regulated FoxO target genes, such as microsomal triacylglycerol transfer protein (MTP), glucose-6-phosphatase, apolipoprotein C-III (apoC-III) and insulin-like growth factor binding-protein 1 in the liver, as well as reduced total plasma levels of cholesterol and triacylglycerol in the pig. Transcriptional suppression of FoxO1, FoxO3, apoC-III and MTP by DHA was further confirmed by reporter assays with each promoter construct. Taken together, our study indicates that DHA modulates lipid and glucose homeostasis in part by down-regulating FoxO function. The down-regulation of genes associated with triacylglycerol metabolism and very low density lipoprotein assembly is likely to contribute to the beneficial effects of DHA on the metabolic syndrome.

Keywords: Docosahexaenoic acid (DHA); Forkhead box O transcription factor (FoxO); Lipid/glucose homeostasis; Pigs

1. Introduction

Obesity has rapidly increased to an alarming level and emerged as a prevalent disease, highlighted by a report from the World Health Organization in 2008 showing that approximately 1.5 billion adults (age>20 years) were overweight and at least 500 million adults were obese [1]. Obesity is closely associated with the development of hypertension, glucose intolerance, dyslipidemia and chronic inflammation, collectively known as the metabolic syndrome [2,3]. Despite the past research efforts in obesity and its related metabolic disorders, the underlying mechanisms remain elusive and await further investigation to better understand the nature of these disorders and to search for solutions to this epidemic.

Eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are two major n-3 polyunsaturated fatty acids (PUFAs) found in fish oil [4]. The US Food and Drug Administration has authorized a certified health announcement that a conventional diet containing EPA and DHA may reduce the risk of coronary artery disease [5]. Our previous studies reported that dietary supplementation with either 2% algal DHA oil for 18 days or 10% DHA oil for 2 days decreased the gene expression of sterol regulatory element-binding protein-1c (SREBP-1c) in weaned pig liver [6,7], consistent with studies in other species [8,9]. Reduction in the expression of this lipogenic transcription factor and its target genes related to fatty acid synthesis implies that dietary DHA is beneficial to reduce lipogenic activity in the liver. The effects of various fatty acids on lipid metabolism and metabolic diseases have been extensively studied, but the underlying mechanisms are still unclear.

The forkhead box O (FoxO) gene was initially identified in *Caenorhabditis elegans* as abnormal DAuer Formation-16 regulating metabolism-linked insulin signaling and life span extension [10]. Mammalian FoxO proteins belong to the class O of the forkhead transcription superfamily and consist of FoxO1, FoxO3, FoxO4 and FoxO6. Among them, FoxO1, FoxO3 and FoxO4 have been reported to be involved in diverse functions, including proliferation, apoptosis, differentiation, stress resistance and metabolic responses [11,12]. FoxO proteins are expressed in a variety of tissues, including the brain, heart, lung, liver, ovary, pancreas, prostate, skeletal muscle,

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spleen, thymus and testis [13]. The FoxO-mediated insulin signaling pathway is conserved in *C. elegans*, *Drosophila melanogaster* and mammals [10]. Upon activation, FoxO proteins recognize the FoxO-response element (FRE) and bind to the core DNA sequence, (T/A)(A/T)AACA [14], to activate the transcription of target genes. The insulin-response element [IRE, T(G/A)TTT(T/G)(G/T)] can also be recognized by FoxO due to its sequence homology to FRE. Moreover, insulin activates Akt pathways to phosphorylate (inactivate) FoxO and further inhibit the binding of FoxO and IRE. This inhibition leads to the suppression of gluconeogenesis and triacylglycerol metabolism in the liver [15].

In this study, we supplemented pig feeds with algal-extracted DHA oil and found that consumption of DHA alters the expression of the FoxO transcription factors and genes related to gluconeogenesis and triacylglycerol metabolism. More importantly, our findings link FoxO to the beneficial effects of DHA on lipid/glucose metabolism and shed new light on its potential role in other FoxO-associated physiological processes and diseases.

2. Materials and methods

2.1. Animals and diets

Thirty cross-bred (Landrace×Yorkshire×Duroc) weaned pigs (mean=18.4±0.3 kg) were purchased from a commercial pig farm and housed at the experimental farm of our department. They were allocated by a randomized block design (five males and five females per pen) and allowed to adapt to the control diet for 7 days. The pigs were then fed diets supplemented with 2% (as-fed basis) of beef tallow (BT), soybean oil (SBO) or DHA oil (DHASCO, Martek Biosciences Corp., Columbia, MD, USA) for 30 days with feed and water provided *ad libitum*. The swine feed formulation of nutrient requirements meets the US National Research Council standards (1998). The 2% (wt/wt, as-fed basis) DHA oil supplementation was chosen to mimic the amount of dietary DHA in 10% dietary fish oil addition [6]. The feed formulation and calculated values of diets are listed in Table 1. The animal experiments were approved by the Animal Care and Use Committee of the National Taiwan University.

2.2. Sample collection and preparation

Pigs were weighed each week and before the start of the experiment. At weeks 0, 2, 3 and 4, blood samples were collected from the anterior vena cava using EDTA as anticoagulant after an overnight fasting. After 30 days of feeding, pigs were sacrificed by electrical stunning coupled with exsanguination. Tissues were snap-frozen in liquid nitrogen and stored at −80°C. Plasma was separated by centrifugation (1500*g* for 25 min at 4°C) and stored at −80°C for future analysis.

2.3. Measurement of plasma glucose, triacylglycerol and cholesterol

Total plasma levels of glucose, triacylglycerol and cholesterol were measured in duplicate using commercial kits (Randox Laboratories, Antrim, UK) according to the manufacturer's instructions.

Table 1
Composition of experimental diets

Diet	Content % (wt/wt, as-fed basis)
Corn	54.9
Soybean meal, solvent extracted	30.15
Skimmed milk	10
Oil (soybean, tallow or DHA[a])	2
NaCl iodide	0.35
$CaCO_3$	0.9
$CaHPO_4$	1.2
Vitamin premix	0.25
Mineral premix	0.25
Total	100
Crude protein	20.90%
ME	3271 kcal/kg
Calcium	0.81%
Phosphate	0.64%

ME, metabolizable energy.
[a] DHA oils extracted from algae containing about 44% of DHA.

2.4. Cell culture and differentiation of human and porcine stromal/vascular cells

SK-HEP-1 cells (a human hepatoma cell line) were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek, Israel). Human preadipocytes were isolated from the breast adipose tissue obtained from a woman undergoing mastectomy. All participants gave written permission, and the experiment was approved by the ethics committee of National Taiwan University Hospital. Procedures for human and porcine preadipocyte isolation, cell culture and differentiation were described previously [16,17].The SK-HEP-1 cells and differentiated adipocytes were cultured in DMEM and DMEM/F12, respectively, overnight and then treated with or without a fatty acid, palmitic acid (PA), oleic acid (OA) or DHA, bound to 1% fatty acid-free bovine serum albumin (BSA) for 24 h.

2.5. RNA preparation and gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues and cells by the guanidinium thiocyanate–phenol–chloroform extraction method [18]. Genomic DNA was then removed from the RNA samples by the TURBO-DNase free kit (Applied Biosystems, Foster City, CA, USA) followed by reverse transcription into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCRs with RealQ-PCR Master Mix Kit (Ampliqon, Herlev, Denmark) were performed using a LightCycler 480 Instrument II (Roche Diagnostics, Indianapolis, IN, USA). PCR was performed for 40 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 30 s. Primers used for amplification are listed in Table 2. The relative value for each target gene was normalized to β-actin expression in the same sample. The threshold cycle (Ct) values were obtained, and relative gene expression was calculated using the comparative Ct method [19]. All samples were analyzed in triplicate, and the PCR amplification efficiency was close to 100%. Amplification of specific transcripts was further confirmed by melting-curve analysis and agarose-gel electrophoresis.

2.6. Protein preparation and immunoblotting analysis

Cells were briefly washed twice with phosphate-buffered saline, and protein was extracted using the radioimmunoprecipitation assay buffer buffer. Nuclear protein was extracted using Nuclear Extraction Kit (Cayman Chemicals, Ann Arbor, MI, USA). For immunoblotting analysis, aliquots of 15 μg of protein were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then blotted onto a polyvinylidine fluoride membrane (Perkin Elmer, Norwalk, CT, USA). The FoxO1 primary antibodies were purchased from Cell Signaling (Beverly, MA, USA). β-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and lamin A/C antibody (Cell Signaling) were used as internal controls. β-Actin was used for the loading control in the lysates of total protein, and lamin A/C was used in the lysates of the nuclear protein fraction. The goat anti-rabbit or -mouse secondary antibody coupled to horseradish peroxidase was used in the chemiluminescence procedure (Immobilon Western, Millipore, Billerica, MA, USA). The Western blotting procedure was performed according to the manufacturer's instruction.

2.7. Plasmid construction and luciferase reporter assay

The promoter regions of human FoxO1, FoxO3, apolipoprotein C-III (apoC-III) and microsomal triglyceride transfer protein (MTP) were amplified from human genomic DNA by PCR (AccuPrime, Invitrogen, Carlsbad, CA, USA). Cloning primers are listed in Table 3. About 1 kb of promoter region was cloned. After sequence verification, PCR products were cloned into the pGL3-Basic vector (Promega, Madison, WI, USA) for promoter activity analysis. SK-HEP-1 cells in 96-well plates were cotransfected with 250 ng of promoter constructs and 25 ng of Renilla luciferase control vector (pRL-TK, Promega) using jetPEI DNA transfection reagents (Polyplus, Illkirch, France) for 24 h before the treatment with 100 μM of DHA for 24 h.

2.8. Statistical analysis

Data were expressed as means±S.E. Results of two groups were compared by the Student's *t* test. Statistical significance among three or more different experimental groups was determined by one-way analysis of variance. Tukey's test was used to evaluate differences between means (SAS Institute, Cary, NC, USA). *P* values≤.05 were considered statistically significant.

3. Results

3.1. DHA reduced the expression of FoxO3 in human primary adipocytes

To decipher the mechanism underlying the effect of DHA on lipid metabolism, human primary adipocytes were treated with or without DHA for 24 h, and the RNA samples were subjected to microarray analysis. Results showed that DHA regulated a variety of genes

Table 2
List of primers used for qRT-PCR analysis

Gene	Sense (5′->3′)	Antisense (5′->3′)	GenBank accession number
FoxO1	AAGAGCGTGCCCTACTTCAA	TTCCTTCATTCTGCACACGA	NM_214014
FoxO3	GGCTGGAAGAACTCTATC	GTACTTGTTGCTGTTGTC	NM_001135959
SIRT1	CGACGACGACGACGACGAC	AGAACTGGCATGTGAGGCTCTATC	NM_001145750
SIRT2	CCACGCCTGCTCATCAACAAG	CCTCCAGCTCCTTCTTCCATCC	NM_001114271
MTP	GCCAGGTCTTCCAGAGCGAGTG	TGCCGTCCTGAGGTGCTGAATG	NM_214185
PEPCK	TGCGGGATTTCGTGGAGA	GTTGTCATACTTCTTCAGCCTCTT	NM_001123158
IGFBP1	TCACAGCAAACAGTGCGAGAC	CCTCTGACCGCCGTGGAG	NM_001195105
apoC-III	GGTCTCCTGGTGCTCCTG	GCTGATACTGTCGGTTACCC	NM_001002801
G6Pase	CGGCTTTCGGTGCTTGAATGTC	AGGCTGGCGTTGTAGATACTCTG	NM_001113445
β-Actin	GCCAGGTCATCACCATCGG	GTAGAGGTCCTTGCGGATGTC	AY550069
h-FoxO1	CGGAATGACCTCATGGATGGA	TAAGTGTAACCTGCTCACTAACCC	NM_002015
h-FoxO3	GGAACTTCACTGGTGCTAAG	ACTGTCCACTTGCTGAGA	NM_201559
h-β-Actin	GAAGATCAAGATCATTGCTCCTC	CTAAGTCATAGTCCGCCTAGAAG	NM_001101

involved in multiple pathways (Supplemental Fig. S1). Among them, we found that the transcription factor FoxO3 was down-regulated twofold by DHA treatment compared to the control group.

3.2. Dietary DHA inhibited the expression of FoxO in the liver and adipose tissue of weaned pigs

To confirm the effect *in vivo*, pigs were fed diets containing three different oils including BT, SBO and DHA oil for 30 days. Fatty acid composition of plasma, liver and adipose tissue was determined in the end of the experiment. Dietary supplementation of algal DHA oil increased DHA concentration in porcine tissues (Supplemental Table S1–S3), indicating that the dietary DHA oil can be utilized to elevate porcine body DHA deposition. Compared to BT supplementation, supplementation with 2% DHA oil or soybean oil did not significantly affect the body weight gain of weaned pigs within the 30 days. The terminal body weights of pigs fed BT, SBO and DHA for 30 days were 39.1±1.3 kg, 37.2±1.0 kg and 38.7±0.9 kg, respectively. Expression of FoxO1 and FoxO3 in the liver and adipose tissue was significantly decreased in the DHA- compared to BT-supplemented pigs (Fig. 1A). However, dietary SBO compared to BT supplementation decreased the expression of FoxO1 and FoxO3 in the liver, but not in the adipose tissue (Fig. 1B). We also evaluated the effects of dietary fatty acids on the expression of sirtuin1 (SIRT1) and SIRT2 (deacetylating FoxO) in adipose and liver, but found no effect of the dietary supplements (data not shown).

3.3. Dietary supplementation with DHA inhibited the expression of FoxO target genes

To address the effect of dietary supplementation of DHA on FoxO-mediated gene expression, we employed Ingenuity Pathways Analysis (Ingenuity Systems) to screen and select candidate genes associated with lipid and glucose metabolism for further gene expression analysis. Selected genes included insulin-like growth factor-binding protein 1 (IGFBP1), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), MTP and apoC-III. We found that, except for PEPCK, hepatic expression of these genes was decreased in DHA-supplemented pigs, whereas dietary SBO had a similar but generally weaker effect than dietary DHA (Fig. 2).

Table 3
List of primers used for promoter construction

Gene	Sense (5′->3′)	Antisense (5′->3′)
FoxO1	TCTCTAACTGCGCTCCTGAG	CTGCTGCCTGTTGAATGTGG
FoxO3	CTAGGTTGAGGCGCCCTGCG	CGATGGAGCCGCGAGCGAAT
MTP	CATTCAAATAGCCCTTTGCTG	AGAAGTGGACTCTTTCAATGG
apoC-III	GGCGCCCTCATCCTGCCATC	TGGAGCAGCTGCCTCTAGGGAT

3.4. Dietary supplementation with DHA reduced plasma levels of triacylglycerol and cholesterol

We measured the plasma levels of triacylglycerol and cholesterol in blood samples collected during the treatment period and found that supplementation with dietary DHA or SBO compared to BT

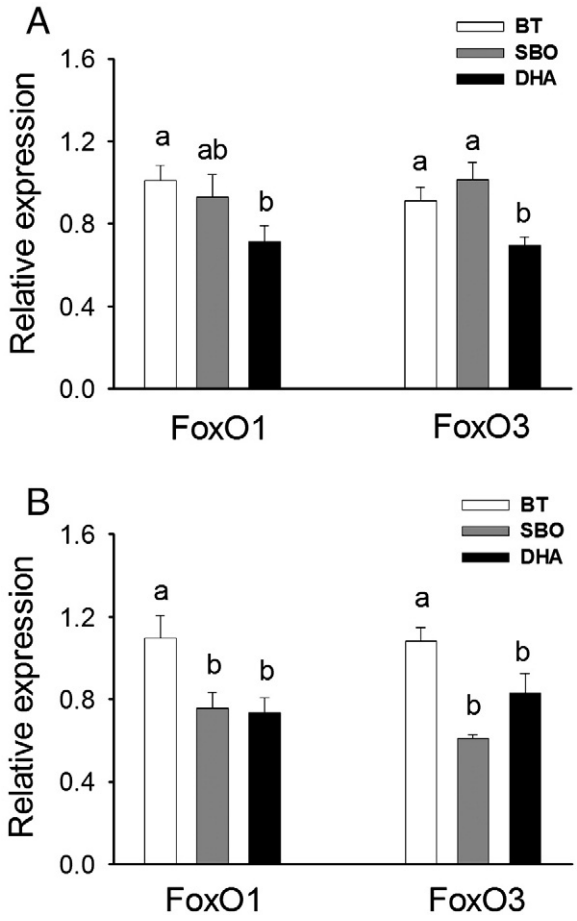

Fig. 1. Effects of dietary treatments on the expression of FoxO1 and FoxO3 in adipose tissue and liver of weaned pigs. The expression of FoxO1 and FoxO3 in the adipose tissue (A) and liver (B) of weaned piglets fed for 30 days with 2% of SBO, BT or DHA oil was analyzed by qRT-PCR as described in the text. Data were expressed as relative means±S.E., $n=7$. Different superscripts indicate significant difference ($P\leq.05$).

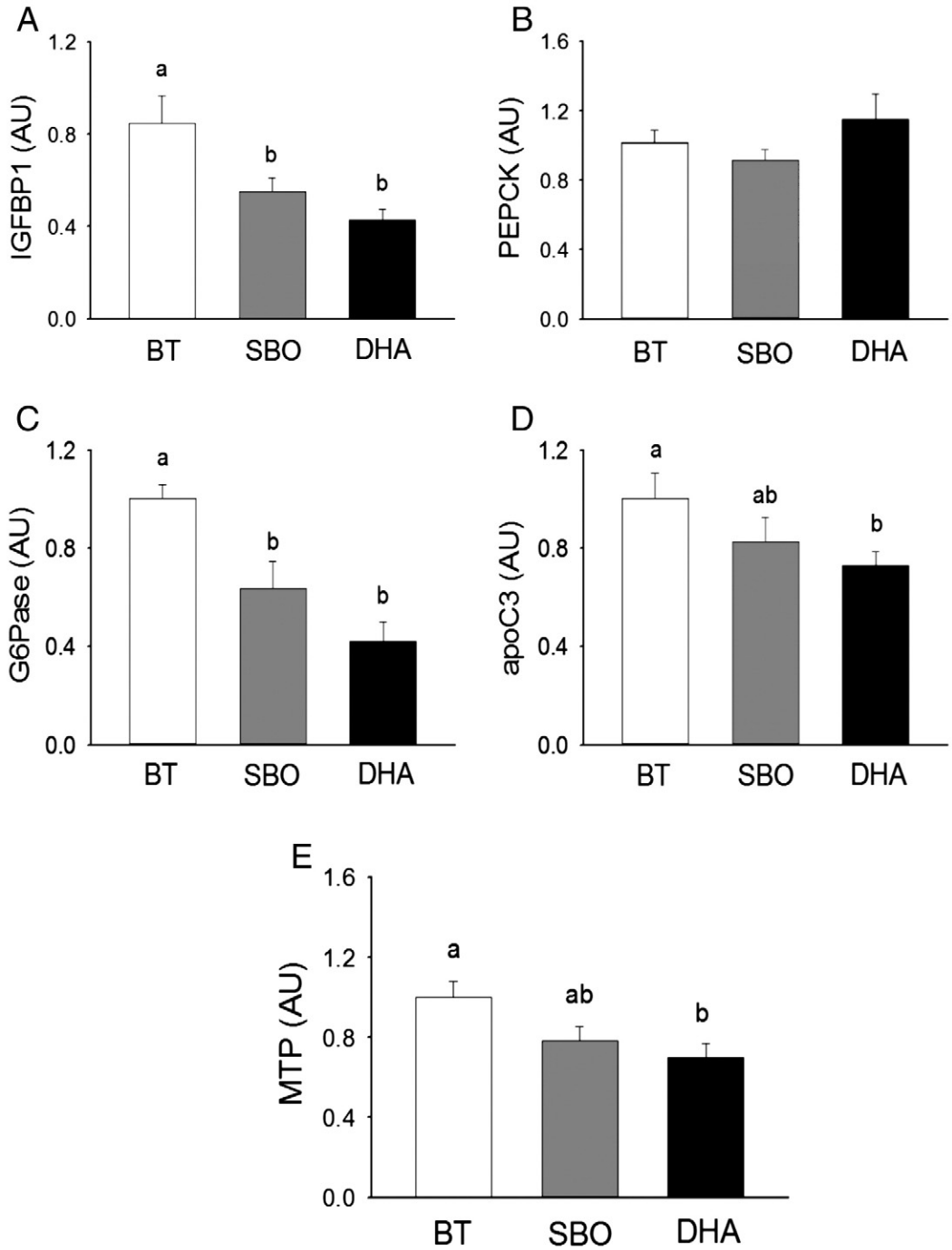

Fig. 2. Effects of dietary treatments on the expression of lipid/glucose metabolism-associated genes in the liver of the weaned pigs. The expression of (A) IGFBP1, (B) PEPCK, (C) G6Pase, (D) MTP and (E) apoC-III in the liver of weaned piglets fed for 30 days with 2% of SBO, BT or DHA oil, respectively, was analyzed by qRT-PCR with gene-specific primers and normalized with β-actin. Data were expressed as relative mean values (arbitrary unit)±S.E., $n=7$. Different letters indicate the significant difference ($P\leq.05$).

significantly decreased the triacylglycerol levels beginning at week 2 (Fig. 3A). Moreover, plasma cholesterol was significantly decreased after 2 weeks of SBO or DHA feeding (Fig. 3B). At week 3, the effect of DHA was more pronounced than that of SBO on plasma triacylglycerol and cholesterol.

3.5. DHA inhibited FoxO expression in hepatoma cells and human and porcine adipocytes

To further confirm the results *in vivo*, we investigated the effect of DHA on FoxO expression in human hepatoma cells, SK-HEP1 and primary adipocytes. One hundred micromolars of DHA significantly decreased the expression of FoxO1 and FoxO3 mRNA in the human and porcine matured adipocytes (Fig. 4A). BT contains large amounts of PA and OA. To clarify whether saturated or monounsaturated fatty acid induces or DHA reduces FoxO expression, we treated SK-HEP-1 cells with BSA control, PA, OA or DHA. DHA decreased the FoxO1 protein content in total cell lysate and in nuclear extracts compared to the control, PA or OA treatments (Fig. 4B). However, PA or OA did not affect FoxO mRNA expression. Moreover, in the reporter assay, we found that DHA treatment also reduced the promoter activities of FoxO1, FoxO3, apoC-III and MTP genes in SK-HEP-1 cells transiently

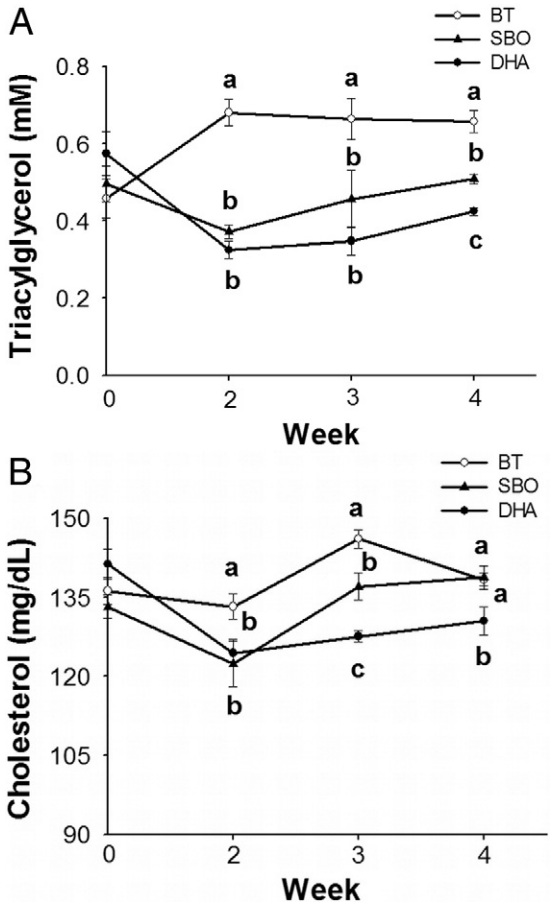

Fig. 3. Effects of a dietary supplementation of fats and oils on plasma triacylglycerol (A) and cholesterol (B) levels. Data were expressed as means±S.E., $n=7$. Different superscripts indicate a statistical significance ($P\leq.05$).

transfected with individual promoter constructs, as measured by luciferase activity (Fig. 4C).

4. Discussion

Dietary fatty acids have been shown to affect lipid metabolism leading to the modification of plasma lipid profiles and body fat deposition [20]. We also demonstrated that in weaned pigs, dietary supplementation with 2% algal DHA oil for 18 days markedly modified the fatty acid composition and gene expression profiles in the liver, muscle and adipose tissue [6]. In terms of the underlying mechanism, transcription factors such as SREBP-1c [21,22], carbohydrate responsive element-binding protein [23], peroxisome proliferator-activated receptor (PPAR) [24,25], liver X receptor [26], nuclear factor-κB [27] and hepatocyte nuclear factor 4 [28] have been reported to mediate the metabolic response of PUFA, including DHA. As in other species, we previously showed that DHA decreases the expression of SREBP-1c in the liver and primary adipocytes in pigs [7,29]. Intriguingly, our current study demonstrates for the first time that FoxO transcription factors FoxO1 and FoxO3 are also negatively regulated by DHA, leading to the down-regulation of their target genes associated with lipid metabolism, as well as the plasma levels of triacylglycerol and cholesterol. This highlights the diverse targets of n-3 PUFA and sheds light on their potential roles in regulating other FoxO-associated processes and diseases in FoxO-expressed tissues. Moreover, whether other members of the forkhead superfamily are targets of DHA is worthy for further investigation.

Because, in mouse embryos and adults, FoxO1, FoxO3a and FoxO4 were found to be abundantly expressed in the muscle, adipose tissue and liver [30], we proceeded to test the hypothesis that fatty acids regulate the expression of FoxOs in these tissues and, indeed, found that dietary DHA, but not BT, decreased the expression of FoxO1 and FoxO3 genes in the liver and adipose tissues, as observed in the *in vitro* system. DHA reduced but PA or OA did not affect FoxO expression. To verify whether such regulation is at the transcriptional level, we performed the luciferase reporter assay with the promoter regions of human FoxO1 and FoxO3 (including their target genes, apoC-III and MTP) and confirmed the gene expression results in the feeding experiment. The mechanisms mediating this regulation need to be further revealed.

In the liver, FoxO1 binds to insulin-response elements to increase gluconeogenesis and triacylglycerol metabolism, and this response is negatively regulated by insulin [15]. G6Pase and PEPCK are two key enzymes responsible for gluconeogenesis induced by fasting and insulin deficiency/resistance, but reduced by feeding or administration of insulin [31]. The involvement of FoxO1 in these processes is demonstrated by increased expressions of PEPCK and G6Pase in the liver of mice constitutively expressing an active form of FoxO1 mutant, resulting in the elevation of fasting plasma levels of glucose and insulin, and impaired glucose tolerance [32]. The increased PEPCK expression is, however, not consistently observed [33], and the mechanism underlying the regulation of PEPCK by FoxO remains elusive. Previous studies indicate that DHA increases PEPCK mRNA in rat adipose tissue and in 3T3-F442A adipocytes, but not in H4IIE hepatoma cells [34]. We also found that dietary DHA had no effect on porcine hepatic PEPCK mRNA expression, but decreased G6Pase mRNA expression, as observed in HepG2 cells, wherein PUFA inhibits the transcriptional activity of the G6Pase promoter [28]. However, in our study, the decreased G6Pase mRNA did not result in altered glucose levels (data not shown), consistent with another previous report in mice fed fish oil [35]. Species differences or tissue-specific metabolic regulation of PEPCK (liver versus adipose tissue) may account for the discrepancy among these studies.

FoxO1 has also been shown to regulate triacylglycerol metabolism through apoC-III and MTP. For example, hepatic FoxO1 expression was elevated, along with increased nuclear localization, in the liver of diabetic *NOD* and *db*/*db* mice [36]. This elevation increases hepatic apoC-III expression, concomitant with increased plasma triacylglycerol levels and impaired glucose tolerance in mice [36]. Regarding MTP, it serves as the rate-limiting enzyme for hepatic very low density lipoprotein (VLDL)–triacylglycerol assembly and has been shown to be stimulated by FoxO1, but inhibited by insulin [37]. Constitutive activation of FoxO1 expression enhances MTP expression, augments VLDL production and elevates plasma triacylglycerol levels [37]. These experiments indicate that FoxO1 plays an important role in triacylglycerol synthesis and its deregulation may be associated with the elevation of triacylglycerol metabolism and insulin resistance in the pathology of diabetic hypertriacylglycerolemia. This is consistent with our current observation that weaned pigs fed diets containing 2% DHA oil exhibited decreased expression of apoC-III and MTP and lowered plasma triacylglycerol concentrations. As a matter of fact, we found that DHA also decreased the promoter activities of human FoxO1, FoxO3, apoC-III and MTP genes, as in pigs. In line with these results, the null mutation of human subject with half expression of apoC-III has a profound effect on lowering the fasting and postprandial serum triacylglycerol and low-density lipoprotein cholesterol and elevating the levels of high-density lipoprotein cholesterol [38]. Moreover, the single nucleotide polymorphism of IRE on apoC-III promoter mediates the effect of n-3 PUFA-lowered apoC-III expression [39]. Therefore, our results suggest that DHA affects apoC-III expression by negatively regulating FoxO binding to the IRE.

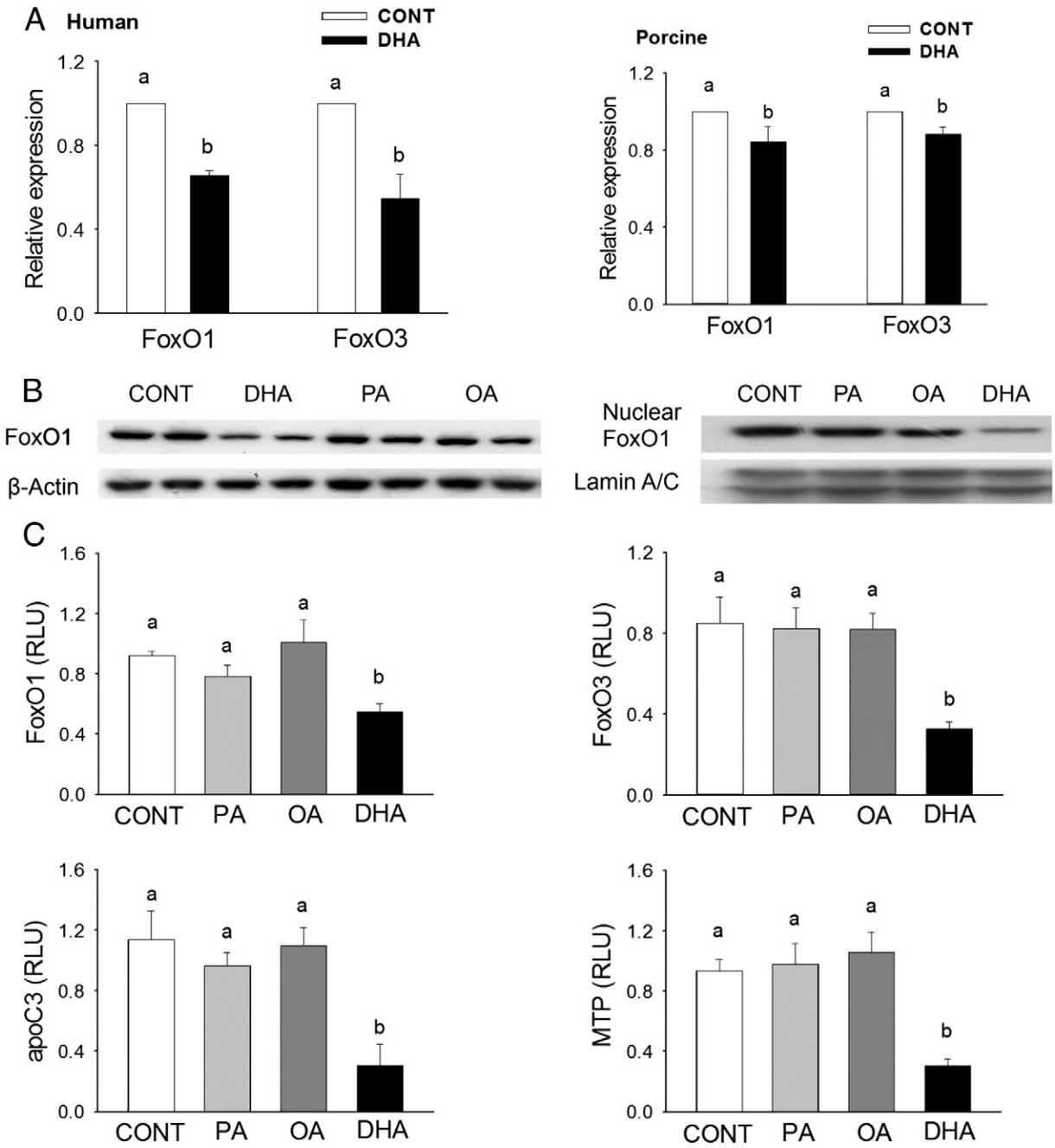

Fig. 4. Effects of fatty acids on the expression of FoxO and its target genes in SK-HEP-1 hepatic cells and primary adipocytes. (A) Human and porcine primary adipocytes were treated with 100 μM of DHA for 24 h, and the FoxO1 and FoxO3 mRNA levels were analyzed by qRT-PCR. (B) Western blotting analysis of protein contents of FoxO1 in the total cell lysates and nuclear extracts of SK-HEP-1 cells treated with 100 μM of PA, OA or DHA bound to 1% BSA for 24 h. The control cells (CONT) were treated with 1% BSA. β-Actin and lamin A/C were used for the control of protein loading. (C) Luciferase assay of promoter activities of FoxO1, FoxO3, apoC-III and MTP genes in SK-HEP-1 cells treated as in (B). The values (relative light units, RLU) were normalized to transfection efficiency using the Renilla luciferase activity. Data were expressed as means±S.E. (n=3) with arbitrary units. Different superscripts indicate statistical significance (P≤.05).

FoxOs are expressed at low levels in preadipocytes, but increased with differentiation. However, constitutively overexpressed nuclear FoxO1 inhibits the differentiation of 3T3-F442A adipocytes [40]. It has been speculated that such effect is due to the antagonistic interaction between FoxO1 and PPARγ. FoxO1 has been found to decrease the interaction between PPARγ/retinoid X receptor complexes and the DNA-response elements [41], and thus, the down-regulation of FoxO1 expression enhances PPARγ activity. Furthermore, FoxO has been shown to repress PPARγ1 and PPARγ2 promoter activity by direct interacting with the PPARγ-binding elements [42]. Therefore, the inhibitory effect of DHA on FoxO1 function observed here may be explained by the competition between FoxO1 and PPARγ for target promoter. It may also due to the fact that DHA is a potent ligand for porcine PPARγ activation [43,44]. However, the underlying mechanism awaits further investigation.

Elevation of plasma free fatty acids (FFA) and triacylglycerol is an indication of increased risks for the metabolic syndrome [45]. Excessive hepatic glucose production is also considered to be a contributing factor for fasting hyperglycemia in diabetes [46]. Dietary EPA and DHA have been reported to reduce insulin resistance in

rodents [47,48]. A meta-analysis indicates that the use of fish oil containing EPA and DHA in clinical non-insulin- and insulin-dependent diabetes mellitus subjects lowers the triacylglycerol levels by almost 30% [49]. Moreover, numerous studies indicate that dietary fish oil prevents dyslipidemia and hyperinsulinemia in rats fed a high-sucrose diet to induce metabolic syndrome [50,51]. The effect of n-3 PUFA is likely to be explained by the activation of fatty acid oxidation and PPARγ to reduce plasma levels of FFA and triacylglycerol [52]. Our previous reports indicate that DHA activates protein kinase A to induce hepatic serum amyloid A expression, resulting in increased lipolysis and reduced triacylglycerol accumulation [16,53]. These data imply that fish oil and DHA are beneficial in preventing and treating dyslipidemia in diabetes. This current report suggests a new aspect that DHA negatively mediates FoxO to influence triacylglycerol metabolism. This is consistent with current alternatives to ameliorate type 2 diabetes by down-regulating FoxO function.

There are more evidences that FoxOs are involved in the development of obesity-related diseases. First, reducing FoxO1 in both the liver and white adipose tissue by using an antisense oligonucleotide-mediated approach has been demonstrated to improve glucose tolerance and both hepatic and peripheral insulin action in mice with diet-induced obesity [54]. Second, haploinsufficiency of FoxO1, which reduces hepatic expression of gluconeogenic genes, improves insulin sensitivity in insulin receptor haploinsufficient mice [33]. Third, FoxO1 haploinsufficiency has been shown to protect mice against high-fat-diet-induced insulin resistance with enhanced effects of antidiabetes drugs of the PPARγ agonist, thiazolidinedione, in adipose tissues [55]. Furthermore, a FoxO1 inhibitor, AS1842856, was identified and shown to improve glucose tolerance by oral administration in diabetic *db*/*db* mice [56]. Our current study demonstrating the negative effect of DHA on FoxO expression in porcine liver and adipose tissues implicates the potential use of DHA as a nutritional intervention to modulate FoxO activity to reduced obesity related diseases. We speculate that this finding provides a link between DHA and FoxO transcription factors to affect its target genes involved in not only gluconeogenesis and triacylglycerol metabolism, but also other biological processes, including cell proliferation, apoptosis, stress resistance and aging.

In conclusion, the current study demonstrated that dietary DHA decreased not only the expression of FoxO1 and FoxO3, but also the expression of hepatic FoxO target genes related to gluconeogenesis and triacylglycerol synthesis in the porcine liver and in human hepatic cells, thereby changing the overall lipid metabolism. We propose that the beneficial roles of DHA in preventing the metabolic syndrome or improving insulin resistance may result partially from the down-regulation of FoxO and FoxO target genes. The beneficial effects of DHA on other FoxO-associated diseases may share the same mechanism, so that DHA can be potentially useful as a therapeutic alternative for preventing and treating related diseases.

Acknowledgments

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Appendix A. Supplementary data

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