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Activation of neuregulin-4 in adipocytes improves metabolic health by enhancing adipose tissue angiogenesis

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ABSTRACT

Obesity often causes systemic metabolic disorders in close association with adipose tissue dysfunction. Adipose tissue contains well-developed vasculatures, and obesity mediates vascular rarefaction that causes hypoxia and triggers inflammation in adipose tissue. Adipose tissue-derived neuregulin-4 (Nrg4) is an immerging factor that is critically involved in metabolic homeostasis. We recently identified that Nrg4 is an angiogenic adipokine that plays an important role in maintaining adipose tissue vasculature. Here, we further validated its beneficial role in metabolic health primarily by enhancing adipose tissue angiogenesis. Targeted activation of Nrg4 in adipocytes improved metabolic health in mice under both normal and high fat dietary condition without changes in body weight. Activation of Nrg4 increased blood vessels in white adipose tissue, and ameliorated adipose tissue hypoxia under obese condition. Of note, inhibition of angiogenesis by sugen-treatment abolished the beneficial effects of Nrg4 on systemic metabolic health. Furthermore, targeted inhibition of Nrg4-ErbB signaling in adipose tissue vasculature using prohibitin binding peptide-conjugated nanocarrier abrogated the enhanced adipose tissue angiogenesis, and canceled the improved metabolic health induced by Nrg4 activation. These data further support a crucial role of Nrg4 in maintaining systemic metabolic homeostasis at least partially through enhancing adipose tissue angiogenesis.

1. Introduction

The epidemic of obesity is a serious concern because of its deleterious effects on health. Adipose tissue (AT) pathologically expands in obesity, which leads to adipocyte dysfunction and consequent metabolic disorders. Although detailed molecular mechanisms remain to be elucidated, obesity-related stresses such as chronic inflammation, endoplasmic reticulum stress, and oxidative stress in AT appears to play a primary role in obesity-related adipocyte dysfunction [1–3]. These stresses affect various genes expression in adipocytes, which collectively impair AT functions and systemic metabolic homeostasis. Reduction in adiponectin represents the dysregulated genes in white AT (WAT), and we recently identified that Fam13a, which plays a crucial role in maintaining insulin signaling in adipocytes, is substantially reduced in obesity, resulting in exacerbated insulin resistance in WAT [4]. In addition, obesity causes vascular rarefaction in both white and brown AT (BAT) [5,6]. Since enhancing AT angiogenesis counteracts obesity and related metabolic disorders, AT angiogenesis is one of important factors involved in systemic metabolic homeostasis [5,7–11].

Neuregulin-4 (Nrg4) is an emerging multifunctional adipokine that plays crucial roles in maintaining systemic metabolic

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homeostasis. Nrg4, a member of neuregulins belonging to the EGF family of extracellular ligands, is highly expressed in BAT and WAT, and shows various biological functions such as promoting neurite outgrowth in neuron cells, inhibiting lipogenesis in hepatocytes, enhancing angiogenic capacities in endothelial cells (ECs), and increasing glucose uptake in adipocytes [12–15]. Genetic loss of Nrg4 impaired metabolic health in association with reduced blood vessels in AT even under normal dietary condition, while it further deteriorated obesity-related metabolic disorders in association with exacerbated hepatosteatosis [13,14]. In contrast, gain of function study revealed that Nrg4 ameliorated obesity-related metabolic disorders in association with promoted fuel oxidation and preserved adipokine profiles in AT [16]. However, whether and how activated Nrg4 affects AT vasculatures that are crucially involved in the fuel oxidation and adipokine production remained to be elucidated. Here, we revealed a critical role of Nrg4-mediated AT angiogenesis in preserving metabolic health.

2. Materials and methods

2.1. Materials

Antibody for phospho-ErbB4 (#4757) was purchased from Cell Signaling Technology. Antibody for ErbB4 (#sc-283) was purchased from Santa Cruz Biotechnology. Sugen (SU-5416) was purchased from Sigma-Aldrich. AST-1306 was obtained from Selleck Chemicals.

2.2. Quantitative RT-PCR

Quantitation of mRNA expression levels was performed as reported previously [17]. Total RNA was extracted using QIAzol (Invitrogen) followed by purification with RNeasy Lipd Tissue Mini Kit (QIAGEN). cDNA was synthesized from ~1 μg of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (TAKARA). PCR reactions were prepared using FastStart SYBR Green Master (Roche Applied Science) followed by the real time PCR analysis using LightCycler96 (Roche Applied Science). The mRNA levels of target genes relative to 18 S were analyzed.

2.3. Nanoparticle preparation

The prohibitin targeting peptide-conjugated PEG-lipid (PTP-PEG_{5k}-DSPE) was prepared as previously described [18,19].

AST-1306 (ErbB1/4 inhibitor) was dissolved in DMSO at 1 mg/ml. Egg yolk phosphatidylcholine (EPC) and cholesterol (Chol) were dissolved in anhydrous EtOH at 100 mM PEG_{2k}-DSPE was dissolved in water at 25 mM. Mixture of 333 μl of 100 mM EPC, 167 μl of 100 mM Chol, 20 μl of 25 mM PEG_{2k}-DSPE and 500 μl of 1 mg/ml AST-1306 were prepared in a glass tube. The drug/lipids/solvent mixture was then injected rapidly into 10 ml of PBS through a fine needle under condition of mechanical stirring at 800 rpm to form lipid nanoparticles. To modify the surface of lipid nanoparticles with the targeting ligand having specificity to ECs of WAT, 62.5 μl of 5 mM PTP-PEG_{5k}-DSPE in water was added to the liposome dispersion under continuous stirring and the resulted dispersion was further stirred for 1 h at 37 °C. The liposome suspension was then passed three times through two polycarbonate membranes (pore size: 200 nm) for particle size regulation, using a Mini-Extruder (Avanti Polar Lipids, Inc.), and dialyzed in a Spectra/Por dialysis membrane (MWCO: 100 kDa) against PBS for 48 h to remove unencapsulated drugs and organic solvents. When we prepare the empty-liposomes, 500 μl of DMSO was mixed with the lipids, instead of the drug solution. The average size and ζ-potentials measured by dynamic light scattering using a Malvern Zetasizer (Malvern Instruments, Ltd.) were 97.0 ± 8.3 nm and -0.7 ± 1.5 mV for the drug-loaded liposomes, while those were 102.9 ± 10.5 and 0.7 ± 0.5 mV for the empty ones, respectively.

The small aliquot of liposome suspensions before and after dialysis were diluted with DMSO at 1: 1 vol ratio, and then the UV absorbance was measured using a UV-VIS spectrophotometer (Beckman Coulter, Inc.) at 352 nm. The percentages of drug encapsulation in liposomes was calculated according to the following formula:

$$\% \text{ encapsulation} = (D_1 - E_1) / (D_0 - E_0) \text{ x } 100$$

where D_1 and E_1 are the absorbance values of the dialyzed drug-loaded and empty liposomes, respectively, whereas D_0 and E_0 are the values of the pre-dialyzed ones. The encapsulation of AST-1306 in liposomes was $88.5 \pm 11.1\%$.

2.4. Metabolic measurements

The Insulin and glucose tolerance tests (ITT and ipGTT) were performed as previously reported [20,21]. After fasting for 6 h, D-glucose at 1.5 g/kg was intraperitoneally administered for ipGTT. For the ITT, mice were given human insulin at 1 IU/kg by subcutaneous injection in the absence of fasting. Mice fed an NCD at the age of 19–21-week or 19–21-week-old mice fed an HFD for 12–14 weeks were regularly used. Blood glucose was measured by the glucose oxidase method (Johnson & Johnson K.K.). Visceral perigonadal WAT and interscaplar BAT were used for all of the analysis for WAT and BAT, respectively.

2.5. Animal study

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kobe Pharmaceutical University. Transgenic mice that overexpressed Nrg4 in adipocytes (aP2-Nrg4-Tg) were generated. The plasmid containing aP2-promotor and SV40-poly A was obtained from Addgene (plasmid 11424). The aP2-Nrg4-Tg mice were propagated as heterozygous Tg animals by breeding with wild-type C57BL6 mice.

Mice were fed either an NCD (containing 23.1% protein and 5.1% fat) or HFD (Oriental Bio #HFD-60) containing 35% fat, 25.3% carbohydrate and 23% protein with *ad libitum* access to water and food. For the HFD feeding, mice at the age of 6–7 weeks old were maintained on an HFD for up to 16 weeks.

For sugen (SU-5416) treatment, sugen was subcutaneously administered at a dose of 10 mg/kg three times a week for 4 weeks.

For blocking ErbB1/4 signaling in the WAT vasculature, nanoparticles in which a prohibitin targeting peptide-conjugated PEG-lipid was incorporated on the surface carrying AST-1306 were intravenously administered at a dose of 0.25 mg/kg twice a week for 4 weeks. Empty nanoparticles was injected into mice of control group in parallel.

2.6. Statistical analysis

All data are presented as mean ± S.E. Differences between groups were analyzed using two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Targeted activation of Nrg4 in adipocytes enhances AT angiogenesis and preserves metabolic health in mice

We previously reported that targeted deletion of Nrg4 caused

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