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Eicosapentaenoic acid upregulates VEGF-A through both GPR120 and PPARγ mediated pathways in 3T3-L1 adipocytes

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ABSTRACT

Vascular endothelial growth factor-A (VEGF-A) released from adipocytes promotes angiogenesis; and thereby ameliorates the local hypoxia-induced adipose inflammation and insulin resistance. Here, we newly found that eicosapentaenoic acid (EPA) upregulated both mRNA expression and release of VEGF-A in mature 3T3-L1 adipocytes. Silencing mRNA of G-protein coupled receptor 120 (GPR120) and specific inhibition of peroxisome proliferator-activated receptor γ (PPARγ) by GW9662 respectively attenuated the EPA-induced augmentation of VEGF-A release by adipocytes. Furthermore, transfection of GPR120 gene alone and PPARγ gene alone to HEK293 cells respectively increased the promoter activity of VEGF-A as assessed by luciferase reporter assay, which was further augmented when both genes were co-transfected. Promoter deletion analysis and chromatin immunoprecipitation assay revealed that co-transfection of GPR120 enhanced EPA-induced PPARγ binding to PPAR-response element in VEGF-A promoter region. Thus, by the synchronized activation of a membrane receptor GRP120 and a nuclear receptor PPARγ, EPA enhances VEGF-A production in adipocytes.

1. Introduction

The dysregulation of macro- and micro-vasculature development in adipose tissue has recently emerged as a major pathophysiological mediator that underlies adipose tissue dysfunction in obesity (Elias et al., 2012; Halberg et al., 2009; Sun et al., 2011, 2012), the so called adiposopathy (Bays, 2011). In times of nutritional excess, adipose tissues expand and store the extra calories in a safer way (Evans et al., 2004). Here adipocytes synchronize the process by secreting various metabolically active and vasoactive adipocytokines (Lazar, 2005). In particular, the proangiogenic adipocytokine, vascular endothelial growth factor-A (VEGF-A), plays a critical role to maintain the demand–supply balance of oxygen and nutrients in growing adipose tissues by facilitating local vascular development (Elias et al., 2012; Sun et al., 2012). However, in chronic obesity, adipocytes often fail to produce enough amount of VEGF-A to form appropriate capillary network (Halberg et al., 2009). This insufficient perfusion of adipose tissue results in hypoxia and inflammation, thereby predisposes the adiposopathy (Bays, 2011; Halberg et al., 2009).

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor and the master regulator of adipocytes regulating hundreds of genes (Lefterova et al., 2008). Activation of PPARγ in adipocytes, despite increasing body fat mass, improves adipocyte function (Lehrke and Lazar, 2005). Interestingly, in addition to the canonical hypoxia response pathway and hypoxia inducible factor-1α (HIF-1α) (Forsythe et al., 1996), PPARγ also enhances VEGF-A expression (Chintalgattu et al., 2007; Emoto et al., 2001). Functional PPAR-response element (PPRE) has been identified in the human VEGF-A promoter region (Peeters et al., 2005). Therefore, it is highly plausible that activated PPARγ could directly bind to the PPRE in the

Abbreviations: BHT, 2,6-di-tert-butyl-4-hydroxytoluene; Bim, bisindolylmaleimide I; ChIP, chromatin immunoprecipitation; Cyto, cytoplasm; DHA, docohexaenoic acid; EPA, eicosapentaenoic acid; Go, Gö6983; GPR120, G-protein coupled receptor 120; GW, GW9662; HIF-1α, hypoxia inducible factor-1α; LDH, lactate dehydrogenase; LY, LY294002; Nu, nucleus; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, PPAR-response element; VEGF-A, vascular endothelial growth factor-A; ω3-PUFAs, ω-3 fatty acids.

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promoter of VEGF-A to initiate transcription of this gene (Ricote and Glass, 2007). Thus, the mechanisms for the amelioration of adipose tissue function by the activated PPARγ possibly include the restoration of angiogenesis.

Some long-chain polyunsaturated ω-3 fatty acids (ω3-PUFAs) act as natural ligands of PPARγ (Edwards and O'Flaherty, 2008), and modulate many PPARγ's target genes (Kalupahana et al., 2011). Indeed, diets enriched with ω3-PUFAs show protective effects against metabolic abnormalities (Oh and Olefsky, 2012). Particularly the eicosapentaenoic acid (EPA) and docohexaenoic acid (DHA) improve adipose tissue function by increasing production of adiponectin (Neschen et al., 2006) and decreasing production of monocyte chemotactic protein-1 and plasminogen activator inhibitor-1 (Kalupahana et al., 2010). However, no data exist regarding the effects of ω3-PUFAs on VEGF-A through direct PPARγ activation.

G protein-coupled receptors, residing in the cell membrane, respond to various extracellular stimuli and trigger cascades of intracellular signal transduction (Talukdar et al., 2011). Among them, the GPR120 in adipocytes has recently been reported to sense and respond to the ω3-PUFAs by coupling with Gαq/11 subunit of G proteins that stimulates protein kinase C (PKC) and MAP kinase, which subsequently activates phosphatidylinositol 3-kinase (PI3K)-Akt pathway enhancing glucose uptake (Oh et al., 2010). Thus, the GPR120-mediated cascade also contributes to the attenuation of the adiposopathy by the ω3-PUFAs (Ichimura et al., 2012; Oh et al., 2010). However, whether this process involves VEGF-A upregulation remains to be shown.

In the present study, we examined the effects of EPA in adipocytes on expression and release of VEGF-A and their mechanisms, especially focusing on relative contribution of PPARγ and GPR120 pathways.

2. Materials and methods

2.1. Cell culture, induction of adipogenesis and interventions

3T3-L1 preadipocytes (JCRB Cell Bank, Tsukuba, Ibaraki, Japan) were cultured and induced to differentiate to adipocytes following our previous report with slight modifications (Hasan et al., 2011, 2014). Briefly, cells were grown in DMEM (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Nichirei Biosciences, Tokyo, Japan) and 1% penicillin–streptomycin–glutamine (Invitrogen, Grand Island, NY, USA) until they became confluent. Two days later, adipogenic induction was given using 0.5 mM IBMX, 1 μM dexamethasone and 170 nM insulin (all from Sigma-Aldrich) for 2 days, followed by 170 nM insulin for next 4 days. At this point most of the cells showed lipid droplets, confirming successful adipogenesis. Then they were grown in DMEM containing FBS and the antibiotics. Adipocytes grown for 8–10 days after adipogenic induction were used in the study. HEK293 cells (JCRB Cell Bank, Japan) were also grown in DMEM containing 10% FBS and the antibiotics.

These cells were treated with varying concentrations of peroxide free EPA (Cayman Chemicals, Ann Arbor, MI, USA) following a previous study (Yeop Han et al., 2010) with slight modification. Briefly, EPA dissolved in ethanol was first mixed with NaOH (both are from Wako Pure Chemical Industries, Osaka, Japan), then conjugated with fatty-acid-free BSA (Sigma-Aldrich) and were heated to 45 °C for 15 min; followed by mixing with culture medium by continuous agitation in a 37 °C shaker for 1 h. For fatty acid treatment, serum free DMEM with antibiotics was used. The molar ratio of EPA to BSA was 2.8:1. Equal volume of ethanol was similarly processed and used as control. According to the manufacturer, to protect autoxidation, the antioxidant BHT (2,6-di-tert-butyl-4-hydroxytoluene) was premixed with the peroxide free EPA. Equal amount of BHT (Cayman Chemicals) alone had no effect on VEGF-A release in 3T3-L1 adipocytes (Fig. S1).

Where indicated, the mature adipocytes were pretreated with 10 μM Gö6983, 10 μM bisindolylmaleimide I or 50 μM LY294002 in serum free medium containing 0.4% BSA and antibiotics to block PKCζ, PKCε or PI3K, respectively for 1 h (Fernández-Galilea et al., 2012). PPARγ activity was blocked overnight using 10 μM GW9662 (all from Cayman Chemicals). Controls were treated with equal volume of DMSO (Wako Pure Chemical Industries). Following the blocking, the cells were treated with ethanol or EPA with these inhibitors or DMSO for the indicated times.

2.2. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was assessed to indirectly measure the cell viability. Culture medium was collected after 24 h of intervention, and amount of LDH released in the medium was measured using LDH cytotoxicity assay kit (Cayman Chemicals) according to the manufacturer's instruction.

2.3. Quantification of VEGF-A release in the medium

Culture medium after the interventions was preserved at –80 °C until use. The concentration of VEGF-A in the medium was determined and defined as the release using an enzyme-linked immunosorbent assay kit (mouse VEGF assay kit, Immuno-Biological Laboratories, Gunma, Japan).

2.4. RNA isolation and qRT PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) as we have reported previously (Hasan et al., 2014). Quantitative PCR was carried out by real-time PCR in a Step ONE Plus Real time PCR system (Life Technologies, Tokyo, Japan) using inventoried *VEGF-A* and *SLC2A1* primers (Mm01281449_m1 and Mm00441480_m1, respectively; Life Technologies) with Premix Ex Taq (Probe qPCR), ROX plus (Takara Bio, Tokyo, Japan), and the primers reported previously for *HIF-1α* (Regazzetti et al., 2010), and *cyclophilin B* (Kosteli et al., 2010) with Fast SYBR Green Master Mix (Applied Biosystems). The mRNA expression is presented as fold change over the control groups.

2.5. Western blotting

Cells were lysed with RIPA buffer (Thermo Fisher Scientific, Rockford, IL, USA). Western blotting was performed using conventional methods as described previously (Hasan et al., 2014). Briefly, after SDS–PAGE electrophoresis proteins were transferred to an Immobilon-P membrane (Millipore, County Cork, Ireland), and where indicated, probed with an anti-VEGF (1:200; sc-1836, Santa Cruz Biotechnology, Dallas, TX, USA), anti-GPR120 (1:400; sc-99105, Santa Cruz Biotechnology), anti-HIF-1α (1:400; NB100-105SS, Novus Biologicals, Littleton, CO, USA), anti-β-actin (1:1000; A5316, Sigma-Aldrich), or anti-Lamin A/C (1:1000; 4777, Cell signaling Technology, Danvers, MA, USA) antibody. The amounts of the proteins were quantified by densitometry using ImageJ software version 1.47g (Rasband, 1997), and the band intensities were standardized to that of anti-β-actin or anti-Lamin A.

2.6. siRNA mediated knockdown

3T3-L1 adipocytes grown in 100 mm dishes after 6 days of adipogenic intervention were used for this experiment. Cells were electroporated with siRNA against Gpr120 (Gotoh et al., 2007) or scrambled siRNA (Universal Negative Control, Sigma-Aldrich) as previously described (Fan et al., 2009) with slight modification. Briefly,