RESEARCH

Exenatide mitigates inflammation and hypoxia along with improved angiogenesis in obese fat tissue

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Abstract

Obesity-associated chronic inflammation in adipose tissue is partly attributed to hypoxia with insufficient microcirculation. Previous studies have shown that exenatide, a glucagon-like peptide 1 (GLP-1) receptor agonist, plays an anti-inflammatory role. Here, we investigate its effects on inflammation, hypoxia and microcirculation in white adipose tissue of diet-induced obese (DIO) mice. DIO mice were injected intraperitoneally with exenatide or normal saline for 4 weeks, while mice on chow diet were used as normal controls. The mRNA and protein levels of pro-inflammatory cytokines, hypoxia-induced genes and angiogenic factors were detected. Capillary density was measured by laser confocal microscopy and immunohistochemistry staining. After 4-week exenatide administration, the dramatically elevated pro-inflammatory cytokines in serum and adipose tissue and macrophage infiltration in adipose tissue of DIO mice were significantly reduced. Exenatide also ameliorated expressions of hypoxia-related genes in obese fat tissue. Protein levels of endothelial markers and pro-angiogenic factors including vascular endothelial growth factor and its receptor 2 were augmented in accordance with increased capillary density by exenatide in DIO mice. Our results indicate that inflammation and hypoxia in adipose tissue can be mitigated by GLP-1 receptor agonist potentially via improved angiogenesis and microcirculation in obesity.

Key Words

- ▶ GLP-1
- ▶ obesity
- ▶ inflammation
- ▶ hypoxia
- ▶ angiogenesis

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Introduction

Obesity is widely considered as a state with low-grade chronic inflammation (de Heredia *et al.* 2012). Elevated circulating levels of inflammatory cytokines, such as tumour necrosis factor (TNF)-alpha, interleukin (IL)-6, plasminogen activator inhibitor (PAI) and C-reactive protein (CRP), have been reported in adults with obesity; and the elevated levels of IL-1beta and IL-6 are predictive

of type 2 diabetes (T2D) (Donath & Shoelson 2011). Besides, the discovery that adipose tissue from humans with obesity and obese mice models is infiltrated with increased numbers of macrophages provides a major mechanistic advance into understanding obesity-associated inflammation (Olefsky & Glass 2010). These adipose tissue macrophages are a major source of

pro-inflammatory cytokines such as IL-6 and TNF- α , which can function in a paracrine and potentially an endocrine fashion to cause decreased insulin sensitivity (Weisberg *et al.* 2003).

Experimental evidence has proved that increased expressions of inflammatory genes could be caused by hypoxia (Ye *et al.* 2007), and macrophages were found to predominantly accumulate in hypoxic areas in adipose tissue of obese mice (Rausch *et al.* 2008). These findings indicate that the trigger of obesity-associated inflammation is hypoxia in expanding adipose tissue. Previous studies showed that the physiological basis of adipose tissue hypoxia might be related to reduction in adipose tissue blood flow and capillary density (Ye 2011). Normally, adipose tissue is highly vascularized with multiple capillaries in contact with each adipocyte (Christiaens & Lijnen 2010). The increase in body fat should be accompanied by an increase in vascularization to provide adequate oxygen and nutrients (Nishimura *et al.* 2007). However, it is not always the case. It has been proposed that insufficient neovascularization during the progressive development of obesity may lead to a relative oxygen deficit in adipose tissue (Gealekman *et al.* 2011). A reduction in blood flow may be a result of such angiogenic failure (Karpe *et al.* 2002). In addition, blood perfusion is reduced due to impaired vasomotor function, evidenced by the finding that arterioles isolated from visceral omental fat in subjects with obesity exhibit severely impaired endothelium-dependent vasodilation (Farb *et al.* 2012).

Clinical studies demonstrated that exenatide, a glucagon-like peptide 1 (GLP-1) receptor agonist, improved glycemic control and reduced body weight in patients with T2D (Nauck *et al.* 2007, Zinman *et al.* 2007, Shyangdan *et al.* 2010). Accumulating data from both clinical and animal studies has shown potential anti-inflammatory effect of GLP-1 receptor agonist on the cellular and molecular level (Lee & Jun 2016). A series of studies conducted in patients with obesity showed that exenatide suppressed oxidative stress and the expression of inflammatory mediators including monocyte chemoattractant proteins (MCP)-1, TNF- α and IL-1 β (Wu *et al.* 2011, Chaudhuri *et al.* 2012), but the underlying mechanism remained unknown. Recent studies indicated that GLP-1 acutely recruited muscle microvasculature in skeletal muscles, expanded muscle microvascular surface area and improved insulin's metabolic action in the insulin-resistant state (Chai *et al.* 2012, 2014). Based on the relationship between obesity-related chronic inflammation and hypoxia

caused by insufficient angiogenesis in adipose tissue, we investigated the actions of GLP-1 on inflammation, hypoxia and microvasculature in white adipose tissue of diet-induced obese (DIO) mice.

Materials and methods

Animal study

Seven-week-old male C57BL/6J mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). They were maintained on a 12-h light/dark cycle with free access to food and water. After one-week acclimatization, mice were randomly distributed into two initial groups fed with a standard chow diet (NC, 4% fat wt/wt, Guangdong Medical Laboratory Animal Center) or a high-fat diet (HFD, 34.9% fat wt/wt, D12492, Research Diets) for 12 weeks. The ones with HFD feeding were then further divided into two groups, one group with exenatide treatment (HFD+Exe group) and another one with normal saline as control (HFD group). Exenatide (Eli Lilly) was injected intraperitoneally (24 nmol/kg daily). Mice in the normal control (NC) group were also injected with normal saline every day. After 4 weeks exenatide administration, blood and epididymal fat tissues were collected. Each group consisted of five mice. All protocols and procedures for animal study were approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University.

Tolerance tests and metabolic measurements

The intraperitoneal (i.p.) glucose tolerance test (i.p.GTT) and insulin tolerance test (i.p.ITT) were carried out after 4-week exenatide intervention. For i.p.GTT, mice were fasted overnight and were given 2 g glucose/kg body weight via i.p. injection. For i.p.ITT, mice were fasted for 6 h and were i.p. injected with recombinant human insulin Novolin R (Novo Nordisk) (0.65 U/kg). Tail vein blood glucose level was measured at 0, 30, 60, 90, 120 min both in i.p.GTT and i.p.ITT.

Serum analysis

After 6 h fasting, C57BL/6 mice were killed and blood samples were collected. Serum samples were separated by centrifugation at 4°C and stored at -80°C until measurements were performed. Serum inflammatory adipokines, including IL-6, PAI and TNF- α , were measured using a MENDO-75K kit (Millipore).

Vascular imaging

Epididymal fat staining was performed as previously reported (Xu *et al.* 2012). Mice were killed and the tip portion of epididymal fat was collected under sterile condition and minced into small pieces (2–3 mm) using scissors. The fat tissue pieces were washed with 1× PBS once and then incubated in PBS containing both Griffonia simplicifolia IB4 isolectin Alexa Fluor 488 (40 µg/mL in PBS) (I21411, Invitrogen, Thermo Fisher) to stain endothelial cells and BODIPY 558/568 (5 µM in PBS) (D-3835, Invitrogen, Thermo Fisher) for lipid staining overnight. The samples were washed three times in PBS (5–10 min per time) and fixed with 4% (wt/vol) formaldehyde for 24 h for long-term storage. Samples were kept in 1× PBS during the imaging process.

Confocal microscopy and image analysis

Vascular images were collected using a Zeiss 510 META confocal microscope with appropriate excitation lasers and band pass filters and equipped with a 20× Plan-Apochromat objective lens (NA, 0.8). Image stacks were collected with a frame average of two and a step size of 0.76 µm. At least five randomly selected data stacks were collected for each sample. To quantify the capillary density, the fluorescent signal was collected with confocal microscope at multiple z-planes in the tissue and then to generate 3D reconstructions. Generation of 3D images was accomplished using Imaris (Bitplane, South Windsor, CT, USA) version 6.2. Isosurface renderings of capillaries in each data stack were made using Imaris software (Bitplane) and vessel volume determined with Imaris Measurement Pro. The total vessel volume in each sample was divided by the total tissue volume for the vascular density in each sample.

Quantitative real-time PCR

Total RNA was extracted from the epididymal adipose tissue using a TRIzol reagent (Invitrogen). cDNA was synthesized using the Prime Script RT Reagent Kit (Takara Bio). Real-time qPCR was performed on the LightCycler® 480 System with LightCycle® 480 SYBR Green Master (04897030001, Roche). Fold change of mRNA expression was determined using the $2^{-\Delta\Delta CT}$ method, with all genes normalized to the mouse β -actin. All qPCR primers and their sequence in this study are presented in Table 1.

Table 1 Sequence of primers used for quantitative real-time PCR.

Primers	DNA sequences
<i>Il-6</i> F	5'-CCGGAGAGGAGACTTCACAG-3'
<i>Il-6</i> R	5'-ACAGTGCATCATCGTGTTC-3'
<i>Il-1b</i> F	5'-GCAACTGTTCTGAACTCA-3'
<i>Il-1b</i> R	5'-CTCGGAGCCTGTAGTGACAG-3'
<i>Mcp-1</i> F	5'-TGTTCTCTTAATCCTGCCCA-3'
<i>Mcp-1</i> R	5'-TCTGGACCCATTCTTCTTG-3'
<i>F4/80</i> F	5'-GGAAAGCACCATGTTAGCTGC-3'
<i>F4/80</i> R	5'-CCTCTGGCTGCCAAGTTAATG-3'
<i>Hif-1a</i> F	5'-GATGAGTTCTGAACGTGAAAAGAAAAGT-3'
<i>Hif-1a</i> R	5'-GAAGTTTTCTCACACGTAAATAACTGATGGTG-3'
<i>Vegf</i> F	5'-GAGCAGAAGTCCCATGAAGTGA-3'
<i>Vegf</i> R	5'-CACAGGA CGGCTTGAAGATGT-3'
<i>Vegfr2</i> F	5'-GCTCCGCCCCCAACTCT-3'
<i>Vegfr2</i> R	5'-CTCATAGCCCGCATCTCCACA-3'
<i>Et-1</i> F	5'-TGTCTTGGGAGCCGAATCA-3'
<i>Et-1</i> R	5'-GCTCGTTGTGCGTCAACTTCTGG-3'
<i>Pdgfa</i> F	5'-CAGTGTCAAGGTGGCCAAAGT-3'
<i>Pegfa</i> R	5'-TGGTCTGGGTTTCAAGTTGGA-3'
<i>Pegfb</i> F	5'-TAATGTTGTTCCCTCGTCC-3'
<i>Pegfb</i> R	5'-AAGGCTCTGCACACTTGT-3'
<i>Tgfb</i> F	5'-CGAAGCGGACTACTATGCTAAAGAG-3'
<i>Tgfb</i> R	5'-CGTCAAAAGACAGCCACTCAGG-3'
<i>Pedf</i> F	5'-GTGGGCAACCAAGTTTGACT-3'
<i>Pedf</i> R	5'-AGGGGCAGGAAGAAGATGAT-3'
<i>Cd31</i> F	5'-AGCCAACAGCCATTACGGTTA-3'
<i>Cd31</i> R	5'-AGCCTTCCGTTCTCTTGGTG-3'
<i>Ve-cad</i> F	5'-ATTGGCCTGTGTTTTCGCAC-3'
<i>Ve-cad</i> R	5'-CACAGTGGGGTCACTGCAT-3'
<i>Angpt1</i> F	5'-ACATGGGCAATGTGCCTACA-3'
<i>Angpt1</i> R	5'-TTTGACAGAGCGTTGGTGTTG-3'
<i>Angpt2</i> F	5'-AGTACCAGGTCCAGAACGGA-3'
<i>Angpt2</i> R	5'-ACGGCATTGGACATGTAGGG-3'
<i>Fgf1</i> F	5'-TTATACGGCTCGCAGACACC-3'
<i>Fgf1</i> R	5'-TCTCCGCATGCTTCTTGGAG-3'
<i>Fgf2</i> F	5'-GCTGCTGGCTTCTAAGTGTG-3'
<i>Fgf2</i> R	5'-ACTGCCAGTTCGTTTCAGT-3'
<i>Fgf10</i> F	5'-GCATGTGCGGAGCTACAATC-3'
<i>Fgf10</i> R	5'-TTCATTCTTGGTCCCCTGA-3'
<i>Glut1</i> F	5'-ATGGATCCAGCAGCAAGAG-3'
<i>Glut1</i> R	5'-CCAGTGTATAGCCGAAGTGC-3'
<i>Pdk1</i> F	5'-TGCAAAGTTGGTATATCCAAAGCC-3'
<i>Pdk1</i> R	5'-TGTGCCGTTTCTGATCCTT-3'
<i>Gpx1</i> F	5'-AATGTCGCGTCTCTCTGAGG-3'
<i>Gpx1</i> R	5'-TTGCCATTCTGGTGTCCGAA-3'
<i>Vhl</i> F	5'-GACTACCAAGTGTGCGGAA-3'
<i>Vhl</i> R	5'-CAAGGCTCCTCTCCAGGTG-3'
<i>Adam8</i> F	5'-TAGCCCCTGGACCTCCTTG-3'
<i>Adam8</i> R	5'-CGCTGGTCCCAAGAGCATAG-3'
β -actin F	5'-GCCCTGAGGCTCTTTTCCAG-3'
β -actin R	5'-GCTGGAAGGTGGACAGTGAG-3'

Western blotting

Methods for protein extraction and Western blotting have been described previously (Xu *et al.* 2016). Antibodies against vascular endothelial growth factor (VEGF, sc-507), vascular endothelial growth factor receptor 2

(VEGFR2, sc-504), platelet/endothelial cell adhesion molecule 1 (PECAM-1, sc-28188), insulin receptor substrate-1 (IRS1, sc-7200) were purchased from Santa Cruz Biotechnology. Antibodies against insulin receptor beta (IRbeta, CST#3025), phosphorylation of IRS1 (p-IRS1, CST#3070), Akt kinase (Akt, CST#4691), phosphorylation of Akt (p-Akt, CST#4060), phosphoinositide-3-kinase (PI3K, CST#4249) were purchased from Cell Signaling Technology. Beta-actin (CST#9470, Cell Signaling Technology) served as a loading control.

Immunohistochemistry

The epididymal fat pads were isolated and fixed in neutral buffered formalin, then dehydrated, and embedded in paraffin. Thin tissue slides (5 μ m) were deparaffinized, blocked and incubated overnight at 4°C with a rat anti-mouse F4/80 (sc-71085, Santa Cruz Biotechnology), a mouse anti-mouse hypoxia-inducible factor (HIF)-1 α (NB100-105, Novus Biologicals, Littleton, CO, USA) and a rabbit anti-mouse PECAM-1 antibody (ab28364, Abcam), which was followed by signal amplification using a VECTASTAIN Elite ABC Kit (PK-6102; Vector Laboratories). The reaction was developed by addition of AEC chromogen substrate (AEC Staining kit; Sigma-Aldrich). All sections were mounted and analyzed at 100 $\times$ and 400 $\times$ magnifications using a digitalized LEICA DMI8 microscope. Three to five random microscopic fields were selected in each sample. The expression of F4/80, HIF-1 α and PECAM-1 were analyzed and evaluated by the average optical density (AOD), as measured by ImageJ software.

Statistical analysis

All data are expressed as means $\pm$ s.e. Results were analyzed by student t-test or ANOVA and statistical significance was set at $P < 0.05$.

Results

Diet induced obesity and impaired glucose homeostasis in mice are improved by exenatide

Male C57BL/6J mice were fed with HFD for 12 weeks to develop obesity. As shown, body weight (Fig. 1A), fat mass (Fig. 1B and C) and ratio of fat mass to body weight (Fig. 1D) in HFD-fed mice were significantly higher when compared with NC mice. After a 4-week exenatide

treatment, HFD-challenged mice exhibited significant weight loss and reduced fat mass (Fig. 1A, B, C and D), which were associated with reduced food intake (Fig. 1E). The fasting blood glucose (FBG) level of the HFD-challenged mice was higher than the level of those fed on the chow diet, and presented a significant reduction after exenatide treatment (Fig. 1F). The i.p.GTT and i.p.ITT were conducted after 4 weeks of exenatide administration. The result of i.p.GTT showed that HFD-challenged mice had impaired glucose intolerance, as indicated by the higher glucose levels at all the time points during the test and the significantly larger area under the curve (AUC) value (Fig. 1G). During the i.p.ITT, higher blood glucose levels at time points 90 and 120 min as well as a larger AUC value were observed in HFD-challenged mice (Fig. 1H). After exenatide treatment, the deteriorated glucose tolerance and insulin sensitivity of the DIO mice were both improved (Fig. 1G and H).

Exenatide suppresses obesity associated pro-inflammatory cytokine production and macrophage infiltration in adipose tissue

To examine the diet-induced adipose tissue inflammation, pro-inflammatory factors including IL-6, PAI and TNF- α were measured in serum. Levels of these cytokines were higher in HFD group compared to NC group (Fig. 2A, B and C). And all of them were significantly reduced by exenatide treatment. Meanwhile, relative mRNA levels of *Il-6* and *Il-1b* in epididymal fat of HFD-challenged mice were obviously up-regulated in comparison to NC group (Fig. 2D). However, exenatide treatment significantly down-regulated mRNA expression of these two inflammatory cytokines (Fig. 2D). Since accumulating studies showed that the elevated inflammatory status appeared to originate from infiltrated macrophages, we also tested the mRNA expression of *Mcp-1*, a member of the small inducible chemokine family that played a role in the recruitment of monocytes to sites of injury and infection; and *F4/80*, a classical macrophage-restricted surface. The relative mRNA level of *Mcp-1* in the adipose tissues was drastically increased in HFD mice compared with NC mice, whereas exenatide significantly decreased its expressions (Fig. 2D). Quantitative RT-PCR and immunohistochemistry measurements showed that the mRNA and protein expression of F4/80 in epididymal fat tissues were significantly increased in HFD-challenged mice compared with NC group (Fig. 2D and E), and the protein level of F4/80 was downregulated by exenatide treatment (Fig. 2E and F). Together, these data indicate

Figure 1

Diet induced obesity and impaired glucose homeostasis in mice are improved by exenatide. Mice in the HFD group were randomized to receive exenatide or normal saline for 4 weeks after a 12-week HFD challenge. (A) Body weight, (B) epididymal fat weight, (C) fat mass, (D) fat mass/body weight, (E) food intake, (F) FBG were analyzed among three groups. The fat percentage in the body composition was determined by the ratio of fat mass to body weight. i.p.GTT (G) and i.p.IIT (H) were performed at the end of the intervention with exenatide or normal saline treatment. The area under the curve (AUC) was determined. $n = 5$ mice per group. All values are expressed as the mean $\pm$ s.e.m. * $p < 0.05$ when compared with NC group; # $p < 0.05$ when compared with HFD group.

that obesity is accompanied with chronic low-grade inflammation, and exenatide can broadly suppress pro-inflammatory cytokines in serum and adipose tissue and reduce macrophage infiltration in adipose tissue.

Exenatide alleviates hypoxia in adipose tissue of HFD-challenged mice

It has been widely accepted that hypoxia resulted in inflammation in adipose tissue (Ye *et al.* 2007). To clarify the underlying mechanism of exenatide-ameliorated inflammation, we detected protein levels of HIF-1 α in epididymal fat of C57BL/6 mice. As shown by immunohistochemistry staining, HIF-1 α expression was higher in HFD mice than NC group (Fig. 3A and B), supporting the hypothesis that hypoxia occurred in adipose tissue with obese state. Exenatide obviously reduced the protein level of HIF-1 α in HFD-challenged mice when compared with HFD group (Fig. 3A and B). Similarly, mRNA levels of genes involved in hypoxia pathway, including *Glut1*, *Pdk1*, *Gpx1*, *Vhl* and *Adam8* were significantly higher in HFD group than that in NC group (Fig. 3C). After exenatide treatment, the mRNA levels of *Hif-1a*, *Pdk1*, *Vhl* and *Adam8* were drastically decreased (Fig. 3C). These results indicate that hypoxia in fat tissue of obese status can be reversed by exenatide.

Exenatide promotes angiogenesis and improves insulin sensitivity in adipose tissue of HFD-challenged mice

We further detected the expression of angiogenic growth factors in adipose tissue. As shown, the protein levels of pro-angiogenic factors such as VEGF and VEGFR2 in epididymal fat of obese C57BL/6J mice were significantly increased when compared with NC group (Fig. 4A, B and C). Similarly, the mRNA levels of pro-angiogenic factors including endothelin 1 (*Et-1*), platelet-derived growth factor alpha (*Pdgfa*), platelet-derived growth factor beta (*Pdgfb*) and transforming growth factor beta (*Tgfb*) in adipose tissue of HFD group were up-regulated in the mRNA levels (Fig. 4D) when compared with NC group. These results suggested that angiogenic factors were upregulated in response to hypoxia in obesity. After exenatide treatment, the protein levels of VEGF and VEGFR2 were further elevated in obese mice (Fig. 4A, B and C). Accordingly, the mRNA levels of majority of pro-angiogenic factors, including *Vegf*, *Vegfr2*, *Pdgfb*, angiopoietin 1 (*Angpt1*), *Angpt2*, fibroblast growth factor (*Fgf1*), *Fgf2* and *Fgf10* were drastically upregulated by exenatide in obese mice, while the mRNA level of platelet derived growth factor (*Pedf*), an anti-angiogenic factor, tended to be downregulated in HFD-challenged mice (Fig. 4D). These results indicate that angiogenic factors mainly including VEGF and VEGFR2 are promoted by

Figure 2

Exenatide suppresses obesity associated pro-inflammatory cytokine production and macrophage infiltration in adipose tissue. After 4 weeks exenatide treatment, C57BL/6J mice were killed and blood samples and epididymal fat tissues were collected. Several key pro-inflammatory factors were measured by ELISA and real-time qPCR. (A, B and C) Serum levels of IL-6, PAI and TNF-alpha in epididymal fat tissues. (D) Relative mRNA levels of *Il-6*, *Il-1b*, *Mcp-1* and *F4/80* were tested in epididymal fat tissue respectively. (E) F4/80 immunohistochemistry detecting murine macrophage infiltration in epididymal fat tissue. The upper line shows microphotographs taken under an objective lens (10 $\times$) and the lower line shows those taken under an objective lens (40 $\times$). (F) Quantitative data of F4/80 expression measured by immunohistochemistry. $n = 3-5$ samples per group. All values are expressed as the mean $\pm$ s.e.m. * $p < 0.05$ when compared with NC group; # $p < 0.05$ when compared with HFD group.

exenatide in adipose tissue of DIO mice. We also measured protein level and phosphorylation status of several molecules involved in the insulin signaling pathway. As expected, exenatide significantly upregulated the protein expression of IR, phosphorylation of IRS-1 and AKT, PI3K in adipose tissue of DIO mice (Fig. 4E and F), indicating that insulin signaling pathway was activated. Taken together, we conclude that exenatide helps to regenerate microcirculation, which may contribute to the reversed hypoxia and improved insulin sensitivity in obese fat tissue.

Exenatide ameliorates the impaired microvasculature in adipose tissue of HFD-challenged mice

In order to investigate whether exenatide improved microvasculature of adipose tissue, we conducted vascular imaging in epididymal fat. The images showed that the size of adipocytes in DIO C57BL/6J mice dramatically increased and capillary volume decreased when compared with NC mice (upper, Fig. 5A). This indicated that microvasculature was deficient in the adipose tissue of obese mice. After 4-week exenatide treatment, microvasculature was markedly induced in HFD-challenged mice when compared with those receiving saline injection (upper, Fig. 5A). A clean capillary network was generated and used

to calculate the vascular volume (below, Fig. 5A). Vascular volume was then divided by the adipocytes and total adipose tissue volume for normalization respectively. Consistent with the confocal imaging, the quantitation of vessels showed that capillary density in the epididymal fat of HFD-challenged group was significantly elevated by exenatide (Fig. 5B and C). Furthermore, the capillary was determined by an endothelial cell marker, PECAM-1, in epididymal fat. Western blotting and the corresponding densitometric analysis showed that exenatide augmented protein levels of PECAM-1 in epididymal fat of HFD-challenged mice in comparison with those with saline treatment (Fig. 5D). In accordance with this result, immunohistochemistry analysis showed that, PECAM-1, as distributed in the extracellular matrix of adipocytes, was obviously elevated by exenatide treatment (Fig. 5E and F). All these data indicate that despite the elevated expression of pro-angiogenic factors in adipose tissue of DIO mice, the ability of vasculature construction of them is restricted. However, this restriction can be ameliorated by exenatide.

Discussion

Chronic low-grade inflammation and macrophage infiltration in adipose tissue have been reported to be

Figure 3

Exenatide alleviates hypoxia in adipose tissue of HFD-challenged mice. Total RNA was extracted from the epididymal fat and examined for gene expression. (A) Immunohistochemical detection of HIF-1alpha in epididymal fat tissue. The upper line shows microphotographs taken under an objective lens (10 $\times$) and the lower line shows those taken under an objective lens (40 $\times$). (B) Quantitative data of HIF-1alpha expression measured by immunohistochemistry. (C) Real-time qPCR was used to quantify mRNA for hypoxia response genes as *Hif-1a*, *Glut1*, *Pdk1*, *Gpx1*, *Vhl*, and *Adam8*. $n = 3$ –5 samples per group. All values are expressed as the mean $\pm$ S.E.M. * $p < 0.05$ when compared with NC group; # $p < 0.05$ when compared with HFD group.

closely related to the development of insulin resistance in obesity (de Heredia *et al.* 2012). In the current study, DIO mice manifest higher levels of circulating inflammatory cytokines and their corresponding increased mRNA expressions in adipose tissue including MCP-1, which promotes macrophage infiltration into adipose tissue (Kanda *et al.* 2006). The increase of macrophage infiltration in adipose tissue, which largely correlates with the degree of obesity, is responsible for producing a significantly elevated level of inflammatory cytokines (Weisberg *et al.* 2003). Moreover, our study confirms the existence of hypoxia in adipose tissue under obesity state. It is well-established that hypoxia could induce pro-inflammatory gene expressions (e.g. TNF-alpha, MCP-1) by activating HIF-1alpha (Ye *et al.* 2007). Deletion of HIF-1alpha in adipocytes reduces macrophage infiltration and inflammation in adipose tissue (Kihira *et al.* 2014). HIF-1alpha is also involved in the induction of pro-inflammatory M1-like polarity of macrophages (Fujisaka *et al.* 2013) and the formation of crown-like structures in adipose tissue (Takikawa *et al.* 2016), further enhancing the inflammatory response in obesity.

Regarding to the cause of hypoxia, it is putatively suggested that as adipose tissue mass expands in obesity, clusters of enlarged adipocytes become distant from the vasculature, leading to local pockets of hypoxia (Trayhurn & Wood 2004). As a counter regulatory response to

hypoxia during its expansion, adipose tissue expresses various angiogenic growth factors, such as VEGF, for neovascularization (Wang *et al.* 2007). Despite of the increased pro-angiogenic factors in adipose tissue, the ability of vasculature construction is hampered in obesity, as shown by the HFD induced reduction in capillary density and endothelial cells in our study. It is consistent with previous studies, which have found that capillary density and endothelial cells function failed to meet the demand of adipose tissue growth in obesity (Pang *et al.* 2008, Spencer *et al.* 2011). This failure leads to an impaired blood perfusion and thus microcirculation dysfunction (Hosogai *et al.* 2007, Pasarica *et al.* 2009), which aggravates a local hypoxia in adipose tissue. Recent study also showed that the HIF-1a knock out DIO mice exhibited improved glucose metabolism with less inflammation, less hypoxia and better vasculature development in adipose tissue (Takikawa *et al.* 2016). This indicates that hypoxia inhibits vasculature development in adipose tissue in HFD-induced obesity.

In addition to weight loss and glucose-lowering effects, emerging data suggests that GLP-1-based therapies show anti-inflammatory effects in chronic inflammatory diseases including obesity (Lee & Jun 2016). It was proved that administration of a recombinant adenovirus producing GLP-1 to ob/ob mice reduced the macrophage population and production of TNF-alpha, MCP-1 and

Figure 4

Exenatide promotes angiogenesis and improves insulin sensitivity in adipose tissue of HFD-challenged mice. Protein expression levels of pro-angiogenic factors and molecules involving the insulin signaling pathway in epididymal fat tissues were measured by Western blotting. Key genes related to angiogenesis were detected by real-time qPCR in epididymal fat tissues. (A, B and C) Protein levels of VEGF and VEGFR2 and densitometric analyses of these panels. Beta-actin, loading control. (D) Relative mRNA levels of *Vegf*, *Vegfr2*, *Et-1*, *Pdgfra*, *Pdgfb*, *Tgfb*, *Pedf*, *Angpt1*, *Angpt2*, *Fgf1*, *Fgf2* and *Fgf10* were tested in epididymal fat tissue respectively. (E and F) Protein levels of insulin signaling pathway (IRbeta, p-IRS1, IRS1, p-Akt, Akt, PI3K) and densitometric analyses of these panels. Beta-actin, loading control. $n = 3-4$ samples per group. All values are expressed as the mean $\pm$ s.e.m. * $p < 0.05$ when compared with NC group; # $p < 0.05$ when compared with HFD group.

IL-6 in adipose tissue through inhibiting nuclear factor- κ B pathway (Lee *et al.* 2012). Similarly, the present study shows that exenatide treatment results in a significant reduction in plasma levels of various inflammatory markers and macrophage infiltration in adipose tissue of DIO mice. In addition, obesity associated hypoxia is reversed by exenatide, which may contribute to the reduced inflammatory mediators and the improved insulin sensitivity in adipose tissue.

Our study also reveals that the impairment in neovascularization of obese adipose tissue is alleviated by exenatide, as shown by increased capillary density and endothelial cell marker PECAM-1. Previous study showed that GLP-1 exerted beneficial effects on endothelial cell independently of its action on glucose, lipid or energy metabolism in subjects with T2D (Goyal *et al.* 2010). For example, exenatide stimulated proliferation of human coronary artery endothelial cells through eNOS-, PKA- and PI3K/Akt-dependent pathway via a GLP-1 receptor-dependent mechanism (Erdogdu *et al.* 2010). Besides, exenatide directly improved endothelial dysfunction in isolated aortas from obese rats through the cAMP or AMPK-eNOS pathways (Han *et al.* 2012). The important role of exendin-4 in modulating angiogenesis was also supported by the fact that exenatide directly regulated

proliferation, migration, spouting and tube formation of endothelial cells, thus induced neovascularization (Kang *et al.* 2013). GLP-1 was also reported to be able to protect microvascular endothelial cells against apoptosis by inactivating the PARP-1/iNOS/NO pathway (Liu *et al.* 2011). Recent studies showed that GLP-1 receptor activation could mitigate vascular endothelial senescence (Lei *et al.* 2017, Yang *et al.* 2017) and restore impaired ischemia-induced blood flow recovery and capillary formation in stressed aortas and muscle (Piao *et al.* 2017). This may be partially attributable to the reduction of inflammation and enhancement of adiponectin production (Piao *et al.* 2017, Yang *et al.* 2017). All these studies indicate that GLP-1 ameliorates endothelial dysfunction, which might promote angiogenesis and improve microcirculation to provide sufficient blood flow and oxygen for the expanding adipose tissue in obesity. Our result shows that exenatide significantly restored capillary density and down-regulated HIF-1 α in obese mice. It indicates that exenatide might improve microcirculation and thus alleviate hypoxia in the adipose tissue.

On molecular mechanism, the beneficial effect of exenatide on angiogenesis is shown by the upregulation of pro-angiogenic factor VEGF and its receptor VEGFR2 in adipose tissue in the current study. VEGF exerts its

Figure 5

Exenatide ameliorates the impaired microvasculature in adipose tissue of HFD-challenged mice. Small pieces of epididymal fat tissues were stained with GS-IB4 and BODIPY overnight for blood vessels and adipocytes measurement respectively. Vessels volume and adipocytes volume were determined by confocal microscopy and 3D reconstruction. (A) 3D images of epididymal fat tissues with adipocytes stained in red and vessels in green, as shown in the upper line. After exclusion of adipocytes, capillary density was displayed in the lower line. (B) Vessels/adipocytes volume ratio. (C) Vessels/ total tissue volume ratio. (D) Protein levels and densitometric analyses of PECAM-1 Beta-actin, loading control. (E) Immunohistochemical detection of endothelial marker PECAM-1 in epididymal fat tissue. The upper line shows microphotographs taken under an objective lens (10 $\times$) and the lower line shows those taken under an objective lens (40 $\times$). (F) Quantitative data of PECAM-1 expression measured by immunohistochemistry. $n = 3-5$ samples per group. All values are expressed as the mean $\pm$ S.E.M. $^*p < 0.05$ when compared with NC group; $^{\#}p < 0.05$ when compared with HFD group.

pro-angiogenic action by binding to VEGFR2, a process that is required for adipocyte differentiation and adipose tissue growth (Kinugasa *et al.* 2012). Previous study revealed that GLP-1 markedly enhanced the proliferation of circulating endothelial progenitor cells derived from bone marrow through increasing VEGF generation (Xie *et al.* 2011). Moreover, GLP-1 promoted angiogenesis in human endothelial cells in a dose-dependent manner through the Akt and Src pathways (Aronis *et al.* 2013), both of which had a pivotal role in VEGF-induced angiogenesis (Bir *et al.* 2012). Therefore, VEGF might be a downstream target of GLP-1 and GLP-1 receptor agonist in regulating angiogenesis.

Previous studies showed that GLP-1 infusion potently expanded muscle microvascular surface area and improves insulin resistance (Chai *et al.* 2012, 2014). Our current study find that GLP-1 receptor activation-mediated augmentation of adipose angiogenesis is accompanied

with reversed hypoxia, reduced inflammation in obese adipose tissue, which may contribute to the improvement of insulin sensitivity in white adipose tissue. It will be interesting to examine whether there is any causal effect among these factors. In addition, previous study found that macrophage HIF-1 α negatively regulated vessel formation by suppressing the expression of angiogenic factors, including VEGF, not only in preadipocytes, but also in endothelial cells and adipocytes (Takikawa *et al.* 2016). It will be worth investigating whether exenatide directly acts on macrophage or other cells in obese adipose tissue for the upregulation of angiogenic factors and the improvement of hypoxia.

In conclusion, our study demonstrates that obesity is accompanied with inflammation, and hypoxia along with insufficient neovascularization in adipose tissue is a potential risk factor for this chronic inflammation. Most importantly, GLP-1 receptor agonist exenatide

protects against obesity-related inflammation and hypoxia in adipose tissue, which may contribute to a local improvement of insulin resistance. Moreover, this might be associated with exenatide's positive effect on angiogenesis and microcirculation in adipose tissue. Further research is deserved to investigate how GLP-1 regulates angiogenesis and microcirculation, and whether there is any causal effect among these factors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y X contributed to the immunochemistry analysis, interpretation and discussion of data, and writing and revising of the manuscript. Z C researched data, performed animal studies, analyzed data and wrote the manuscript. H D analyzed data. M C, H L and W X contributed to the revision of the manuscript. J W contributed to the study design, revision of the manuscript, and approval of the version to be submitted. F X contributed to the study design, acquisition of data, data interpretation, manuscript writing and revising and approval of the version to be submitted. F X is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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