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THE THERAPEUTIC ROLE OF LIRAGLUTIDE IN THE MODULATION OF ADIPOSE TISSUE ANGIOGENESIS

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ABSTRACT

Modulation of angiogenesis in adipose tissue (AT) has been suggested as a potential target in preventing AT dysfunction and improving insulin sensitivity. Liraglutide is a glucagon-like peptide-1 (GLP-1) analogue, approved for type 2 diabetes treatment, known for its effects in insulin secretion and sensitivity and lipid homeostasis. However, besides its glucose-lowering properties, Liraglutide has also been suggested to be a modulator of angiogenesis. Thus, our aim was to assess the role of Liraglutide in improving AT angiogenesis and the metabolic outcome. Therefore, we divided 14-week-old Wistar (W) and non-obese type 2 diabetic Goto-Kakizaki (GK) rats into four groups: two groups of W and GK rats injected subcutaneously with Liraglutide (200μg/Kg, twice a day) during 14 days (W Lira; GK Lira) and two control groups comprising W and GK rats with no treatment (W; GK). The systemic metabolic profile and markers of angiogenesis in epididymal adipose tissue (EAT) were evaluated. GK rats showed decreased levels of proangiogenic factors as well as a marked insulin intolerance and a lower activation of the insulin receptor (IR) in EAT when compared to W group. Liraglutide partially improved insulin tolerance and completely restored IR activation in GK rats. Liraglutide treatment also led to a marked weight loss and an improved lipid profile in GK rats. In the EAT of GK rats no significant alterations were observed in the levels of the endothelial cell marker CD31, angiopoietin-2 (Ang-2) and its receptor Tie-2, but a significant increase of vascular endothelial growth factor (VEGF), its receptor VEGFR2 and the hypoxia inducible factor-2α (HIF-2α) was observed. Thus, this study suggests that Liraglutide improves AT angiogenesis which is associated with an improved AT insulin signalling and a better metabolic outcome in type 2 diabetes.

Keywords: Obesity, Type 2 Diabetes, Adipose Tissue, Angiogenesis, Liraglutide.

RESUMO

A modulação da angiogénese do tecido adiposo (TA) tem sido sugerida como alvo terapêutico na prevenção da disfunção do TA e no aumento da sensibilidade à insulina. O Liraglutido, um análogo do peptídeo semelhante a glucagon-1 (GLP-1), aprovado no tratamento da diabetes tipo 2 pela sua ação na secreção e na sensibilidade à insulina e na homeostase lipídica, tem sido igualmente referido como modulador da angiogénese. Assim, o nosso objetivo consistiu na avaliação do papel do Liraglutido na melhoria da angiogénese do TA e do perfil metabólico. Estudámos, por isso, 4 grupos de ratos Wistar (W) e ratos Goto-Kakizaki (GK), um modelo de diabetes tipo 2 não obeso, ambos com 14 semanas de idade: dois grupos, W Lira e GK Lira, constituídos por ratos W e GK tratados com injeções subcutâneas de Liraglutido (200μg/Kg, duas vezes por dia) durante 14 dias e dois grupos controlo, W e GK, formados por ratos W e GK não submetidos a tratamento. Avaliámos o perfil metabólico sistémico e marcadores da angiogénese no tecido adiposo epididimal (TAE). Os ratos GK, relativamente aos ratos W, apresentaram níveis diminuídos dos fatores pro-angiogénicos, uma marcada intolerância à insulina e uma menor ativação do recetor da insulina (RI) no TAE. O Liraglutido induziu um aumento parcial da tolerância à insulina e um aumento significativo da ativação do RI nos ratos GK, assim como uma melhoria no perfil lipídico e uma perda significativa de peso. No TAE dos ratos GK não se verificaram alterações significativas relativas aos níveis do marcador de célula endotelial CD31, da angiopoietina-2 (Ang-2) e do seu recetor Tie-2, mas observou-se um aumento significativo do fator de crescimento endotelial vascular (VEGF), do seu recetor VEGFR2 e do fator induzido por hipóxia-2α (HIF-2α). Por conseguinte, conclui-se que o Liraglutido promove a angiogénese do TA que está associada a um aumento da sinalização da insulina no TA e a um melhor perfil metabólico, na diabetes tipo 2.

Palavras-chave: Obesidade, Diabetes tipo 2, Tecido Adiposo, Angiogénese, Liraglutido.

INTRODUCTION

According to the World Health Organization (WHO), obesity prevalence has increased significantly worldwide, affecting now the adult and juvenile population. Visceral obesity is linked to the development of a chronic low-grade inflammatory state, leading to an increased risk of metabolic syndrome, type 2 diabetes and cardiovascular disease [1]. Metabolic syndrome is defined by American Heart Association as a complex condition including at least 3 of the following metabolic risk factors: increased waist circumference, hypertriglyceridemia, low levels of high density lipoprotein (HDL) cholesterol, hypertension and fasting hyperglycaemia.

Adipose tissue (AT) is a heterogeneous structure which contains many cell types including adipocytes, preadipocytes, immune cells and endothelial cells [2]. Besides being the body's major energy storage compartment, it is also an endocrine organ secreting a broad range of adipokines, including pro-inflammatory, anti-inflammatory, angiogenic and vasoactive factors [2]. AT growth and function depends on a dynamic vascular network which allows oxygen and nutrients supply, waste products removal and the transport of immune cells, adipokines, hormones including insulin and growth factors [3,4]. AT blood flow is then regulated by both vasoactive factors related mechanisms and angiogenesis, which leads to the formation of new sprouts from the pre-existing vessels as an answer to an acute hypoxia [3,4]. Accordingly, angiogenesis and adipogenesis are closely linked and mutually regulated, since an efficient angiogenesis is required during AT expansion in order to prevent the development of chronic hypoxia and AT dysfunction [4].

Obesity-associated AT expansion comprises adipocyte hyperplasia (cell number increase) and hypertrophy (cell size increase) [4,5]. While the first allows a normal and well-adjusted vascular network, some studies suggest that adipocyte hypertrophy is linked to a limited and impaired vascularization, potentially causing permanent hypoxia [4,5]. This may occur by means of adipocyte vascular compression, the formation of aberrant vessels and an inadequate vascular network [4,5]. Furthermore, adipocytes hypertrophy is also associated with the accumulation of secondary products of lipid metabolism and the increase of intracellular levels of free fatty acids (FFA) by means of peroxisome proliferator-activated receptor gamma (PPAR-γ) inhibition [4-6]. The increase of the circulating FFA also occurs, which may result in an ectopic FFA deposition [4-6]. Thus, both lipotoxicity and hypoxia conditions trigger similar inflammatory pathways which will further increase AT dysfunction. This may occur by means of lipolysis, PPAR-γ inhibition, substrate uptake inhibition, alteration of the secretory profile with the increase of pro-inflammatory adipokines and the recruitment of further inflammatory cells in order to enhance angiogenesis [4,6]. Moreover, it may be found insulin signalling inhibition, leading to insulin resistance [4,6].

Actually, previous studies provided evidences supporting the significant role of AT function and angiogenesis in the modulation of insulin sensitivity [6]. Therefore, a reduced blood flow associated to an AT dysfunction and insulin resistance are suggested to be closely related [6].

Insulin resistance is likewise present in type 2 diabetes as well as a beta-cell dysfunction [7]. Methylglyoxal (MG) is a potent glycating agent, derived mainly

endogenously from glucose, fructose and lipid metabolism, which levels are elevated in prediabetic and diabetic patients [8]. Besides inducing oxidative stress, MG is also a strong advanced glycation end products (AGE) precursor, reacting mainly with proteins and DNA and modulating signalling and pro-inflammatory pathways, which may lead to β-cell failure and insulin resistance [8]. Indeed, several studies have correlated insulin resistance to the effect of glycation in the inactivation of insulin receptor (IR) and its pathways and by impairing insulin ability to bind and activate the IR [8].

Liraglutide is a long-lasting glucagon-like peptide-1 receptor (GLP-1R) agonist widely used for type 2 diabetes treatment, based on a glucagon-like peptide-1 (GLP-1) molecule which is resistant to dipeptidyl peptidase-4 (DPP-IV) enzymatic degradation due to its binding to a palmitic acid [9]. Liraglutide mimics therefore the effects of GLP-1, a gastrointestinal incretin hormone, including a weight loss effect, based on a delayed gastric emptying as well as appetite and food intake inhibition, and a glucose lowering effect mainly by the glucose-dependent stimulation of insulin synthesis and secretion and the inhibition of glucagon secretion [9,10].

Regarding the AT, it was observed the GLP-1R expression in adipocytes and Liraglutide was shown to lead to adipogenesis stimulation and AT redistribution with a decrease of total AT areas and ectopic lipid accumulation, reducing thereby lipotoxicity and insulin resistance [10-13].

Several studies have also suggested Liraglutide to be an important modulator and promotor of angiogenesis in both in vitro and in vivo models, improving endothelial

dysfunction [14-16]. Actually, it is known that vascular endothelium expresses numerous GLP-1R and that GLP-1 exerts many vascular protective effects, inducing vasodilation and stimulating endothelial proliferation and angiogenesis [16,17].

Therefore, regarding the strong association between AT angiogenesis and its function as well as the Liraglutide effects in lipid homeostasis, insulin sensitivity and angiogenesis modulation, our goal was to evaluate the role of Liraglutide in improving AT angiogenesis, relating this with the metabolic outcome.

MATERIALS AND METHODS

Animal Models and Maintenance: We studied 14-week-old male Wistar and non-obese type 2 diabetic Goto-Kakizaki rats, from our breeding colonies (Faculty of Medicine, University of Coimbra). Animals were kept under standard ventilation, temperature (22-24ºC), humidity (50-60%) and light (12h light and 12h darkness) with free access to water and food (standard diet A03, SAFE, Barcelona). The experimental protocol was approved by the local Institutional Animal Care and Use Committee and all the procedures were performed by licensed users (FELASA).

Experimental groups: Male 14-week-old Wistar rats were randomly divided in 2 groups (n=7/group): 1) Control Wistar rats maintained with no treatment (W); 2) Wistar rats treated with Liraglutide (W Lira). Male age-matched Goto-Kakizaki rats were also randomly divided in 2 groups (n=4/group): 3) Control Goto-Kakizaki rats maintained with no treatment (GK); 4) Goto-Kakizaki rats treated with Liraglutide (GK Lira).

Liraglutide and isotonic solution administration: To the W and GK control groups it was given a subcutaneous injection of an isotonic solution (NaCl 0.9%), twice a day, during 14 days. W Lira and GK Lira were treated with a subcutaneous injection of 200μg/Kg of Liraglutide, twice a day, during 14 days. Body weight was daily registered in order to do any necessary Liraglutide dose adjustment.

Glycaemic profile and triglycerides levels: Before starting (day 1) and after concluding the treatment with Liraglutide (day 14), fasting glycaemia (6h) and triglycerides levels were measured in the tail vein using reactive test stripes, a glucometer (Glucometer, Bayer,

Germany) and a portable analyser (Accutrend Plus, Roche, Germany), respectively. An intraperitoneal insulin tolerance test (ITT) was also performed in the first and the last day of the treatment: after an intraperitoneal insulin injection (0.25U/Kg) glycaemia was measured 15, 30, 60 and 120 minutes later. The area under the curve (AUC) concerning the intraperitoneal ITT was then calculated.

Adipose tissue collection: After these procedures, animals were sacrificed by cervical displacement and epididymal adipose tissue (EAT) was immediately collected, washed in an isotonic solution (0.9% NaCl) and frozen in liquid nitrogen, being stored at -80ºC.

Western Blotting : EAT sections of 300mg were homogenized in a lysis buffer [25mM Tris, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 10mM PMSF and 40µl/g tissue of proteases inhibitor cocktail (Sigma, USA), pH=7.7]. Homegenates were centrifuged at 14000 rpm for 20 minutes at 4ºC. The supernatant fraction was collected, ultra-sonicated for 10 seconds (70 Hz) and centrifuged again at 14 000 rpm for 15 min at 4ºC. Supernatants were then collected and aliquoted. Protein concentration was determined using the BCA (Bicinchoninic Acid) method (Pierce, USA). Samples were loaded and separated by SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis*)*, with 8% acrylamide gels, being then transferred to PVDF (Polyvinylidene Difluoride) membranes. Membranes were blocked with TBST-0.1% solution [25mM Tris-HCl, 150mM NaCl, 0.1% Tween, pH=7.6] supplemented with 5% of BSA (Bovine Serum Albumin). The membranes were then incubated overnight at 4°C with the primary antibodies and during 2h at room temperature with the secondary antibodies (anti-mouse, GE Healthcare, UK; anti-rabbit and anti-goat, Bio-

Rad, USA). After each procedure, membranes were washed with TBST solution. Membranes were revealed using ECL (Enhanced Chemiluminescence) substrate in a Versadoc system (Bio-Rad, USA) and analysed with the software Image Quant® (Molecular Dynamics, USA). Calnexin was used and quantified as a loading control.

Reagents: Primary antibodies used were directed to Calnexin, VEGFA, CD31 (AB0037, AB0063, AB0112, Sicgen, Portugal), PPAR-γ, VEGFR2, (#2443, #2479, Cell Signaling, USA), p-IR(Y1361), Tie-2, IRβ (sc-324, sc-57342, SantaCruz Biotechnology, USA), Ang-2, GLUT-4, HIF-2α (Ab8452, Ab65267, Ab8365, Abcam, UK), GLP-1R (bs-1559R, Bioss, USA).

Statistical analysis: Results are presented as mean ± SEM per group. Non-parametric Kruskal-Wallis test was used to determine statistical differences between the groups, given the small sample number. It was used SPSS software (IBM, NY, USA) and $p<0.05$ was considered significant.

RESULTS

Table 1: Body weight, fasting glycaemia, serum triglycerides and food consumption in Wistar and GK rats.

Group	W		GK		W Lira		GK Lira	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Body weight (g)	389.4 ±13.2	390.1 ±13.4	323.5 ±5.9 **	312.9 ±2.4 **	399.3 ±9.9	361 ±8.8	320 ±0.8 **	302.5 ±7.3 ***
Fasting Glycaemia (mg/dL)	92.3 ±3.6	84.1 ±2.4	150.5 ±26.1 *	140.5 ±25.3 *	93.1 ±3.7	87.9 ±4.6	175.8 ±42.5 *	115.5 ±5.1 *
Triglycerides (mg/dL)	101 ±4.9	106.1 ±9.0	162.5 ±24.5 *	207.5 ±52.3 *	111 ±7.9	100.3 ±7.8	161.8 ±18.4 *	111.3 ±6.6
Food consumption (g/rat/day)	16.9±0.6		18.3±1.6		11.2 ± 0.8 *		11.6±1.3 *#	

W initial- 14 weeks old Wistar rats; W final- 16 weeks old Wistar rats; GK initial- 14 weeks old Goto-Kakizaki rats; GK final- 16 weeks old Goto-Kakizaki rats; W Lira initial- 14 weeks old Wistar rats before starting the Liraglutide treatment; W Lira final- 16 weeks old Wistar rats after Liraglutide treatment during 14 days; GK Lira initial- 14 weeks old Goto-Kakizaki rats before starting the Liraglutide treatment; GK Lira final- 16 weeks old Goto-Kakizaki rats after Liraglutide treatment during 14 days. Data is presented as mean ± SEM. * Different from W at the same age; # Different from GK at the same age. 1 symbol $p<0.05$; 2 symbols $p<0.01$; 3 symbols $p<0.001$.

Body weight and Food consumption

Despite no significant differences concerning the food consumption, GK rats (GK) had lower body weight than age-matched normal rats (W), which is an intrinsic characteristic of the strain ($p<0.01$) (Table 1).

Liraglutide treatment resulted in a significant decrease in food consumption in both normal (W Lira) and type 2 diabetic (GK Lira) rats, also leading to a body weight reduction in GK rats (GK Lira), through the increase of the difference between GK Lira and W rats, concerning the GK rats body weight before ($p<0.01$) and after ($p<0.001$) Liraglutide administration (Table 1). These results strengthen the already established role of Liraglutide in weight loss and food intake inhibition.

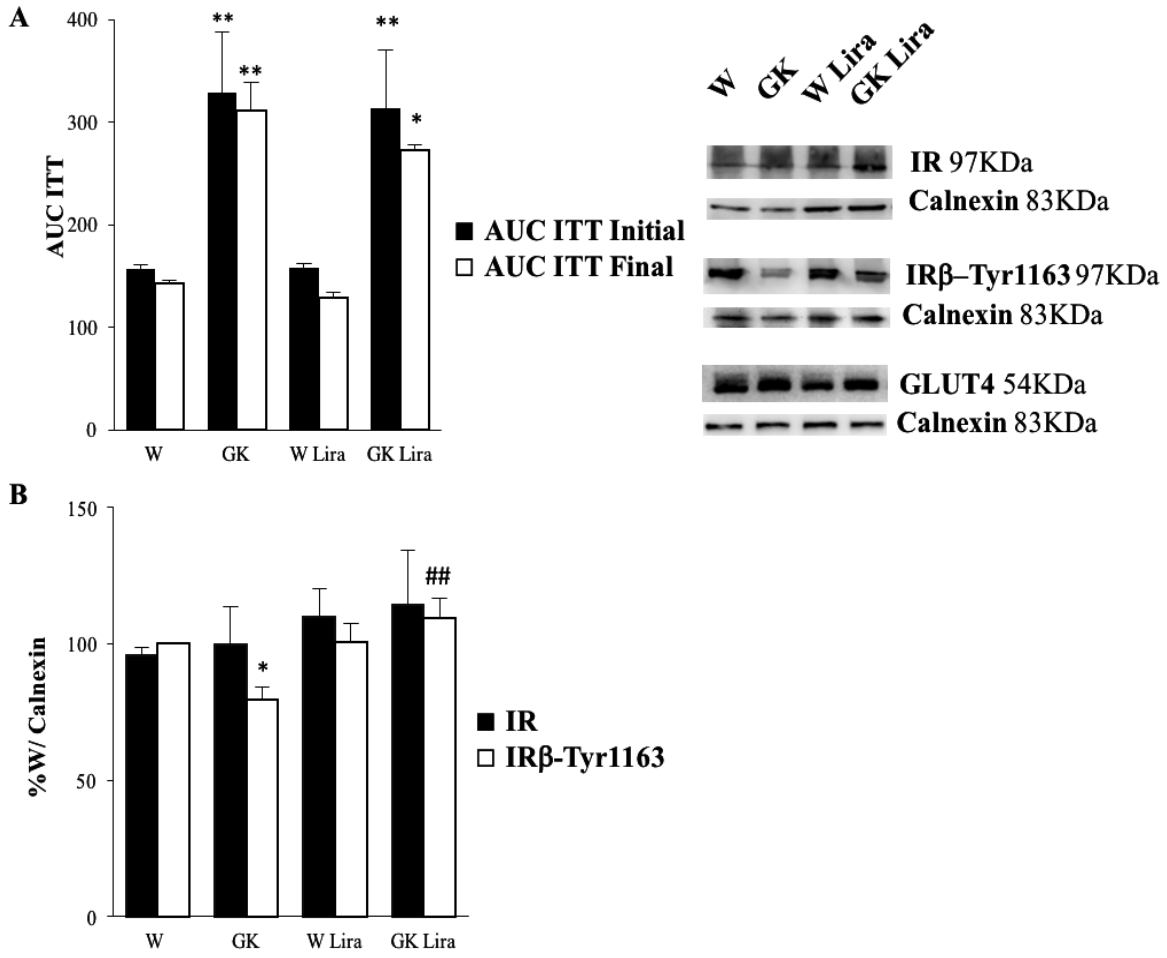

Figure 1: Insulin tolerance determined through AUC of the intraperitoneal ITT before (AUC ITT Initial) and after (AUC ITT Final) Liraglutide administration (A) and EAT levels of total IR (IR) and phosphorylated β-subunit of the IR (IRβ-Tyr1163) (B). Representative Western Blots images of IR, IRβ-Tyr1163 and GLUT-4 are shown with the respective calnexin. W- 16 weeks old Wistar rats; GK- 16 weeks old Goto-Kakizaki rats; W Lira- 16 weeks old Wistar rats after Liraglutide treatment during 14 days; GK Lira- 16 weeks old Goto-Kakizaki rats after Liraglutide treatment during 14 days. Data is presented as mean ± SEM. * Different from W at the same age; # Different from GK at the same age. 1 symbol $p<0.05$; 2 symbols $p<0.01$.

Glycaemic profile, Insulin tolerance and Insulin receptor

As expected, GK group showed higher fasting glycaemia when compared to W group (Table 1). Liraglutide administration did not result in any statistically significant alteration, considering fasting glycaemia, in both normal (W Lira) and diabetic (GK Lira) rats (Table 1). However, a non-statistically decrease is observed in the fasting glycaemia of GK Lira group after the treatment (Table 1).

The AUC referring to the intraperitoneal ITT is directly related to the insulin sensitivity of the insulin-responsive tissues. Regarding the AUC of ITT, whereas similar results were observed in W and W Lira groups, GK group showed a marked insulin intolerance with a higher AUC when compared to W group ($p<0.01$) (Figure 1A). Despite no significant differences were observed between GK and GK Lira groups, the difference in AUC of ITT between diabetic (GK Lira) and normal (W) rats was partially attenuated after Liraglutide administration, with a partial decrease of AUC in the end of the treatment in GK Lira rats ($p<0.05$ vs W), suggesting that Liraglutide treatment partially improved insulin tolerance in diabetic rats (Figure 1A).

In EAT, type 2 diabetic rats (GK) and normal rats (W) showed similar levels of total IR (IR), but GK group revealed lower levels of the phosphorylated, and so activated, β-subunit of the IR (IRβ-Tyr1163) than W group (Figure 1B). No statistically significant alterations whether it concerns total IR or IRβ-Tyr1163 were observed in Liraglutide W-treated rats (W Lira) in relation to W group (Figure 1B). Liraglutide administration in GK rats (GK Lira) did not significantly change total IR levels but caused a marked increase in IRβ-

Tyr1163 when compared to GK group ($p<0.01$), suggesting a Liraglutide action in promoting insulin receptor phosphorylation and activation (Figure 1B).

Regarding the glucose transporter type-4 (GLUT-4), it was not observed any significant alteration in any group (Figure 1).

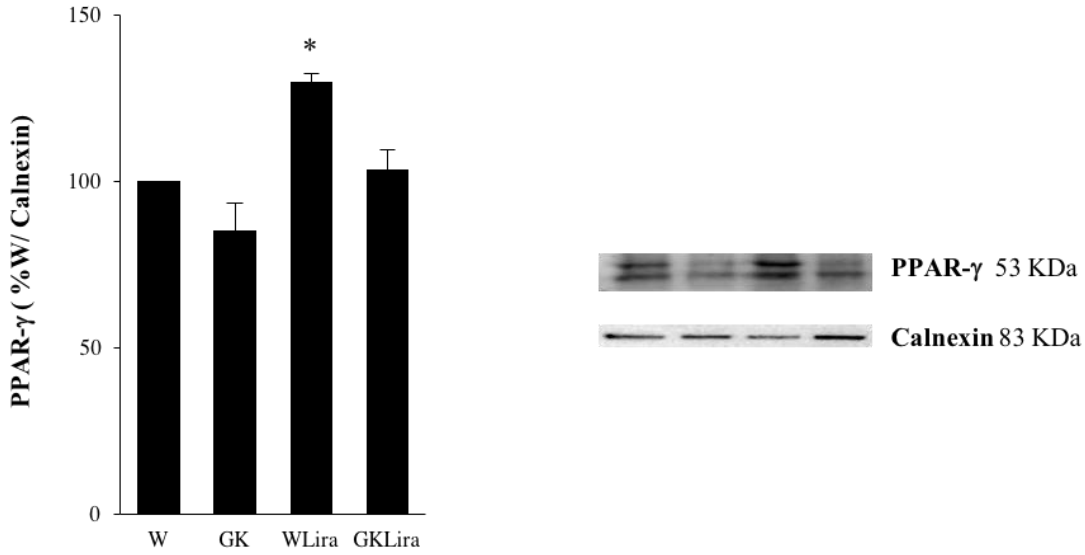

Figure 2: Western Blot detection of PPAR-γ levels in EAT. Representative Western Blots images of PPAR-γ are shown with the respective calnexin. W- 16 weeks old Wistar rats; GK- 16 weeks old Goto-Kakizaki rats; W Lira- 16 weeks old Wistar rats after Liraglutide treatment during 14 days; GK Lira- 16 weeks old Goto-Kakizaki rats after Liraglutide treatment during 14 days. Data is presented as mean ± SEM. * Different from W at the same age. 1 symbol $p<0.05$.

Lipid profile and PPAR-γ

Regarding lipids, GK rats (GK) showed increased serum triglycerides (TG) levels in relation to age-matched controls (W) (Table 1).

Despite W Lira group had similar serum TG levels to W group, in diabetic rats (GK Lira) a significant reduction of serum TG levels was observed after Liraglutide treatment through the attenuation of the difference of TG levels of GK rats before and after the treatment (GK Lira initial vs final) when compared to W group (Table 1).

PPAR-γ, an important marker of AT function due to its involvement in the control of FFA uptake and esterification, was also determined. PPAR-γ expression in AT was significantly increased in Liraglutide-treated Wistar rats (W Lira) when compared to W control rats (W) (Figure 2). It was not observed any other statistically significant difference concerning the other groups, although GK group revealed a tendency to have lower PPAR-γ levels (Figure 2).

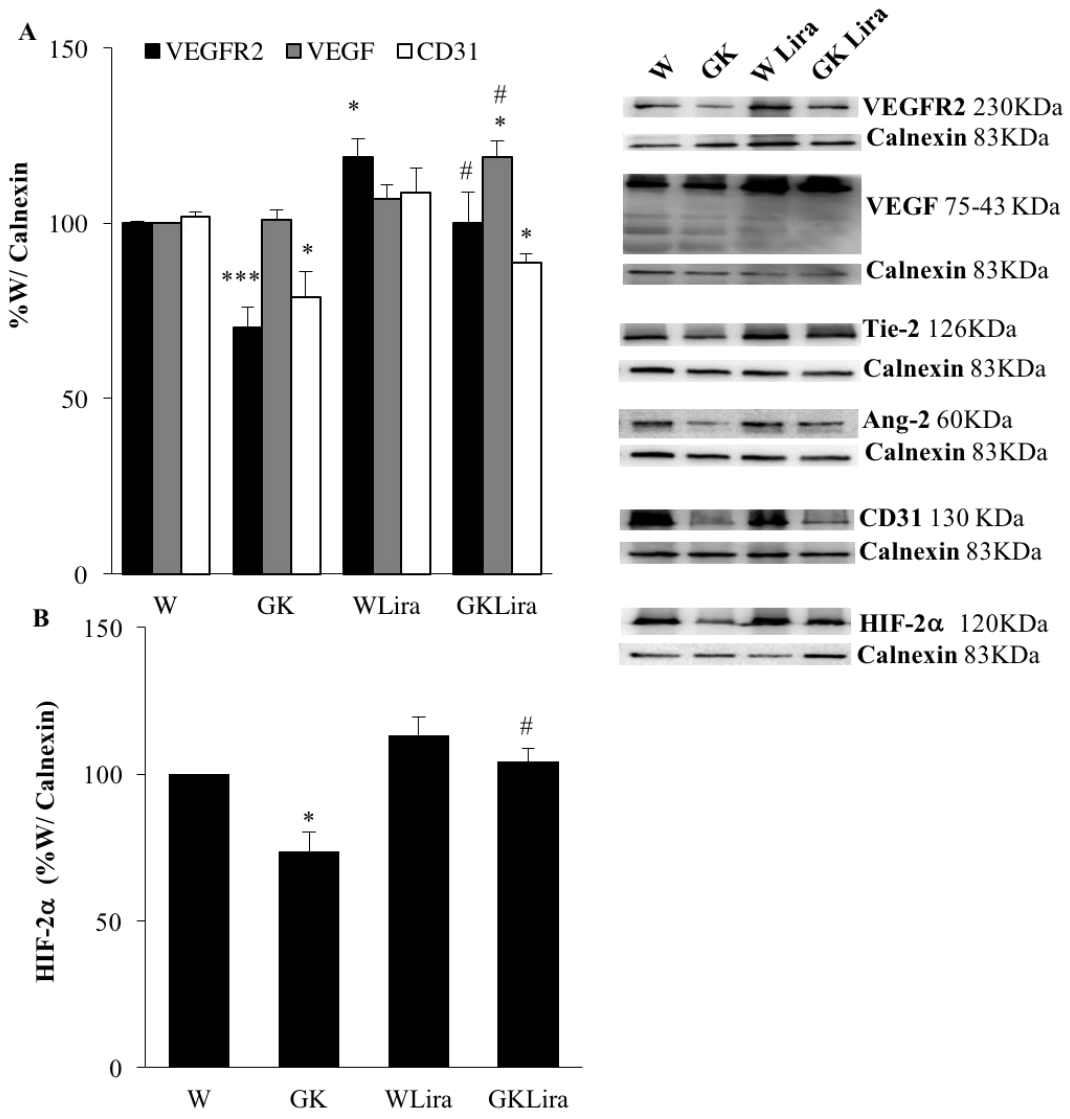

Figure 3: Western Blot detection of VEGFR2, VEGF, CD31 (A) and HIF-2α (B) levels in EAT. Representative Western Blots images of VEGFR2, VEGF, CD31, HIF-2α, Ang-2 and Tie-2 are shown with the respective calnexin. W- 16 weeks old Wistar rats; GK- 16 weeks old Goto-Kakizaki rats; W Lira- 16 weeks old Wistar rats after Liraglutide treatment during 14 days; GK Lira- 16 weeks old Goto-Kakizaki rats after Liraglutide treatment during 14 days. Data is presented as mean ± SEM. * Different from W at the same age; # Different from GK at the same age. 1 symbol $p<0.05$; 3 symbols $p<0.001$.

Angiogenic factors

In order to study the effects of Liraglutide in AT angiogenesis, it was determined EAT levels of the vascular endothelial growth factor (VEGF) and its receptor VEGFR2, the endothelial cell marker CD31, a major transcription factor induced by hypoxia HIF-2α and other angiogenesis key regulator angiopoietin-2 (Ang-2) and its receptor Tie-2 (Figure 3).

Regarding Ang-2 and Tie-2, no statistically significant alterations were observed related to any of the groups (Figure 3).

Type 2 diabetic rats (GK) had significantly decreased VEGFR2 levels as compared to control rats (W) ($p<0.001$), as well as decreased levels of CD31 and HIF-2α ($p<0.05$) (Figure 3A, B). However, GK group had VEGF levels similar to W group (Figure 3A).

Liraglutide administration did not significantly affect VEGF, CD31 or HIF-2α levels but caused a marked increase in VEGFR2 levels in normal rats (W Lira) ($p<0.05$) (Figure 3A, B). Despite no significant changes in CD31 levels in the EAT of diabetic rats after Liraglutide treatment, GK Lira group showed a significant increase of VEGFR2, VEGF and HIF-2α levels as compared to non-treated GK rats (GK) (Figure 3A,B). Thus, these findings suggest that Liraglutide plays an important role in the modulation of AT angiogenesis.

Liraglutide and GLP-1R

Regarding EAT GLP-1R levels, similar results were observed in each group (Figure 4). Liraglutide treatment did not affect GLP-1R levels neither in normal (W Lira) nor diabetic rats (GK Lira), which suggests that Liraglutide actions are independent of GLP-1R levels (Figure 4).

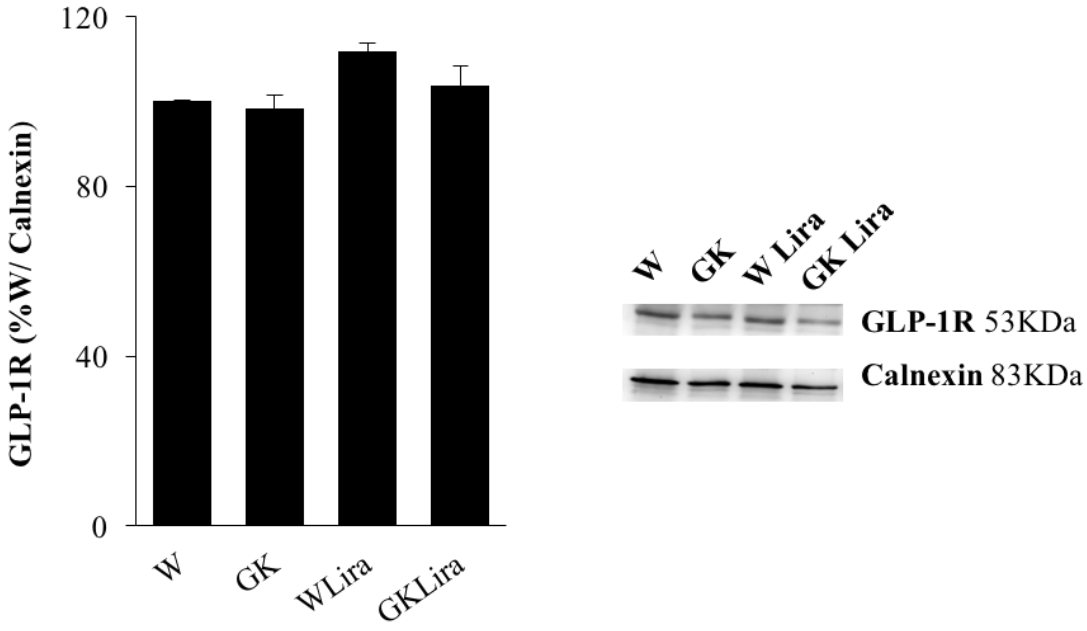

Figure 4: Western Blot detection of GLP-1R levels in EAT. Representative Western Blots images of GLP-1R are shown with the respective calnexin. W- 16 weeks old Wistar rats; GK- 16 weeks old Goto-Kakizaki rats; W Lira- 16 weeks old Wistar rats after Liraglutide treatment during 14 days; GK Lira- 16 weeks old Goto-Kakizaki rats after Liraglutide treatment during 14 days. Data is presented as mean ± SEM.

DISCUSSION

In this study, we investigated the angiogenic properties of the GLP-1 analogue Liraglutide in the EAT in both Wistar rat, a normal animal model used as control, and GK rat, a non-obese type 2 diabetic animal model. We determined the levels of major angiogenic markers (CD31, VEGF, VEGFR2, Ang-2, Tie-2 and HIF-2α) as well as the activation of the IR and systemic metabolic effects of Liraglutide.

GK strain is characterized by an impaired insulin secretion and central and peripheral insulin resistance [18]. Accordingly, a mild fasting hyperglycaemia, a marked insulin intolerance with lower activation of the IR and a lipid dysmetabolism are then type 2 diabetic-like systemic alterations to be expected in GK rats. Furthermore, our group has previously described in young GK rats an increased secretion of leptin, an anorectic hormone produced by AT, which, although the lower body weight when compared to normal rats, is associated with increased abdominal fat [19].

This study reinforces the effectiveness of Liraglutide for weight loss and caloric intake inhibition, which is entirely consistent with previous studies which have correlated the weight loss with a decrease of visceral and subcutaneous AT mass [9,10]. A recent study supports that Liraglutide may reduce the appetite and food intake by central GLP1-R activation of serotonergic neurons having the hypothalamus as a target, an energy homeostasis regulator [20].

Liraglutide is known to enhance systemic and AT insulin sensitivity, leading to improved insulin signalling by promoting adipocyte differentiation, a down-regulation of the inflammatory status and also the PPAR-γ activity, associated to a higher adiponectin secretion [10-13]. Indeed, the results from the present study suggest that Liraglutide treatment improves insulin signalling in EAT of diabetic rats, partially improving insulin tolerance. Moreover, insulin signalling was also enhanced since we observed that the activation of the intracellular

β-subunit of the IR was completely restored after Liraglutide treatment in GK rats. After binding to the extracellular α-subunit of the IR, insulin triggers the auto-phosphorylation of the β-subunit and the IR substrate (IRS) family, leading to insulin signalling pathways which will trigger the translocation of GLUT-4 to the cell membrane [21]. Unexpectedly, after Liraglutide treatment no differences were observed regarding the fasting glycaemia and GLUT-4 EAT levels in both normal and diabetic rats, although we have not studied its translocation to the membrane. On the contrary, a previous study conducted in another diabetic animal model supports that the effect of Liraglutide in improving insulin sensitivity may be due to the upregulation of GLUT-4 [21]. A recent study also revealed that Liraglutide directly stimulates glucose uptake by skeletal muscle cells through GLUT-4 translocation to the cell membrane in an insulin-independent manner, through AMP-activated protein kinase (AMPK) phosphorylation [22].

Regarding the lipid profile, a previous study carried on humans reported an improvement in postprandial lipid metabolism with Liraglutide [23]. Consistently, we observed a significant reduction of serum TG in GK rats after Liraglutide treatment, which might be due to an increased expression of PPAR-γ. It is already established that PPAR-γ activation improves insulin sensitivity and lipid homeostasis by promoting adipogenesis and FFA uptake and esterification in AT, reducing circulating FFA and TG and avoiding therefore ectopic lipid accumulation [4,6]. Moreover, recent studies have demonstrated that AT vascularisation can directly be modulated by PPAR-γ, with an increase of capillary density [4,6]. Surprisingly, Liraglutide only increased PPAR-γ expression in normal rats, without significantly affecting its expression in diabetic rats.

Adipocytes are responsible for the secretion of several pro-angiogenic factors which reflects its ability to self-regulate angiogenesis in AT. Indeed, our results showed decreased levels of proangiogenic mediators namely VEGFR2, CD31 and HIF-2α in type 2 diabetic

rats, which is in accordance with our previous findings of a decreased AT blood supply in older GK rats [19,27].

Nevertheless, the major aim of this study was to determine the effects of Liraglutide in AT angiogenesis and we observed that Liraglutide treatment was able to increase EAT expression of VEGFR2, VEGF and HIF-2α in GK rats. These findings suggest the involvement of Liraglutide in the vascular remodelling process in AT, which is consistent with previous studies showing that Liraglutide significantly improves angiogenesis and exerts a protective effect in the vascular dysfunction [14-16]. Indeed, a recent study supports that Liraglutide promotes angiogenesis and long-term recovery of focal cerebral ischemia in mice through an increased VEGF expression [14]. Liraglutide was also shown to increase angiogenesis of transplanted islets through VEGF up-regulation and to restore the angiogenesis of impaired cultured human umbilical vein endothelial cells (HUVEC), promoting endothelial tube formation in a dose-dependent manner [15,16]. VEGF and its receptors are crucial regulators of vasculogenesis and angiogenesis, promoting the recruitment, proliferation and differentiation of endothelial cells [3,14]. In fact, VEGF and VEGFR2 signalling plays a major role in both physiological and pathological angiogenesis, restoring the capillarization area and vessel density and also improving vessel structure [3,14]. On the other hand, HIF-2α, an important regulator of the hypoxia adaptation, is thought to bind to a specific promoter region of VEGF, which might be linked to increased levels of VEGF activating subsequently VEGFR2 during hypoxia [3,14,24].

Interestingly, whereas VEGF and VEGFR2 levels in AT were increased in GK rats after Liraglutide treatment, no alterations were observed regarding Ang-2 and Tie2, also known for angiogenesis and inflammation modulation. Current data indicate that, under different conditions, Ang-2 can either enhance Tie-2 and induce angiogenesis or can act as a Tie-2 antagonist supressing its downstream targets and causing vessel instability and

regression [3,25]. However, a recent study conducted in mice under a high fat diet observed the pro-angiogenic effects of Ang-2 in AT, along with a reduced inflammatory status and an enhanced insulin sensitivity [25].

In general, our study showed that Liraglutide have more effects in GK rats than in normal rats, possibly because diabetic rats have shown decreased levels of angiogenic factors and a decreased activation of the IR than normal rats.

One limitation to the present study is its short duration, since the exposure time to Liraglutide was only for 14 days, which could explain the modest improvement of the metabolic profile obtained after Liraglutide treatment, mostly concerning the fasting glycaemia and the GLUT-4 expression. We also observed that Liraglutide did not lead to significant changes in CD31 levels, which also might be due to the short duration of our study, adequate to allow the activation of pro-angiogenic mediators being nonetheless insufficient for the formation of a significant vascular network. Further studies with a longer duration are then needed in order to optimize the effects of Liraglutide. Additionally, our sample size is also significantly small which could also justify the current discrepancies.

Altogether, our findings demonstrate that Liraglutide increases AT angiogenesis along with an improved insulin signalling and metabolic outcome. This is in accordance with previous studies which have suggested AT angiogenesis as a potential target and an effective strategy in promoting insulin sensitivity, since an impaired AT angiogenesis could limit adipogenesis through local AT hypoxia and thereby contribute to AT dysfunction, being this a crucial event for insulin resistance [3,4,6]. Accordingly, several studies have also reported the implication of HIF-2α in improving insulin signalling by preventing AT inflammation and increasing its angiogenic ability [24,26].

Our study also suggests that Liraglutide actions are independent of GLP-1R levels since they were not affected. However, we mainly focus on the impact of Liraglutide in AT

angiogenesis and so the molecular mechanism underlying Liraglutide-induced angiogenesis remains therefore to be elucidated.

Recently, we observed that glycation and AGE accumulation in EAT of obese rats contributed to an impaired microvascular network and vascular dysfunction in AT, hampering AT expandability and adaptation, which is likely to lead to AT dysfunction and insulin resistance [27]. Thus, we could hypothesize if the pro-angiogenic effects induced by Liraglutide are related to any modulation or inhibition of the glycation-induced vascular lesions in AT.

Further studies are therefore warranted to provide a better understanding of the molecular mechanisms underlying the promotion of angiogenesis by Liraglutide. Additional studies evaluating the angiogenic effects of Liraglutide should also be performed in obese models, given that angiogenesis and adipogenesis are closely related. Such mechanisms should also be assessed in other fat pads such as subcutaneous AT.

CONCLUSION

Our results suggest that in type 2 diabetes the GLP-1 analogue Liraglutide improves AT angiogenesis, highlighted by the increase of local angiogenic markers levels, and AT insulin signalling which may lead to the enhancement of AT function along with a better metabolic outcome.

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