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Liraglutide demonstrates a therapeutic effect on mitochondrial dysfunction in human SGBS adipocytes *in vitro*

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ABSTRACT

Aims: Liraglutide (LG), a glucagon-like peptide-1 receptor (GLP-1R) agonist, has been shown to improve white adipose tissue mitochondrial metabolism in mice but not in human adipocytes. Therefore, we explored whether LG has therapeutic efficacy in mitochondrial dysfunction in human adipocytes *in vitro*.
Methods: We tested the effects of short-term (ST-LG: 24 h) and long-term (LT-LG: D0-15 days) treatments in human SGBS adipocytes on mitochondrial respiration, mRNA and protein expression. GLP-1R inhibition was investigated by the co-treatment of GLP-1R inhibitor, exendin 9–39 (Ex9-39) and ST-LG treatment. We also explored the ability of ST-LG to alleviate mitochondrial dysfunction induced by tumor necrosis factor-alpha (TNFα).
Results: LT-LG treatment induced the formation of smaller lipid droplets and increased the expression of genes related to lipolysis. Both ST-LG and LT-LG treatments promoted mitochondrial respiration. Additionally, LT-LG treatment increased the expression of a brown adipocyte marker, uncoupling protein 1 (UCP-1), and the markers of mitochondrial biogenesis. Interestingly, ST-LG rescued TNFα-induced defects in mitochondrial energy metabolism and inflammation in SGBS adipocytes.
Conclusion: LG stimulates mitochondrial respiration and biogenesis in human adipocytes, potentially *via* UCP-1-mediated adipocyte browning. Importantly, our study demonstrates for the first time that LG has a therapeutic potential on mitochondrial activity in human adipocytes.

1. Introduction

Obesity is associated with white adipose tissue dysfunction [1], which contributes to the development of whole-body metabolic dysregulation and eventually to the onset of type 2 diabetes [2]. Mitochondrial aberrations provoked by inflammation have been suggested to be one of the key molecular mechanisms promoting white adipose tissue dysfunction [2]. Interestingly, weight loss modifies mitochondrial metabolism in white adipose tissue; bariatric surgery-induced weight loss has more favorable effects on white adipose tissue mitochondrial metabolism than diet-induced weight loss [3–5]. Specifically, bariatric surgery-induced weight loss improves mitochondrial respiratory capacity [6] and the expression of genes involved in mitochondrial oxidative phosphorylation and biogenesis in white adipose tissue [5,7]. By contrast, weight loss through caloric restriction has been shown to down-regulate genes related to mitochondrial metabolism in human white adipose tissue [3–5]. Thus, bariatric surgery-induced weight loss induces more beneficial changes in white adipose tissue mitochondrial function than dieting, indicating that surgery- but not dieting-induced weight loss can restore white adipose tissue mitochondrial function in obesity.

The favorable effects of bariatric surgery on metabolic improvements, such as glucose and lipid homeostasis, insulin sensitivity, and inflammation, are thought to be mediated through gut-secreted

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peptides, like glucagon-like peptide-1 (GLP-1) [8]. GLP-1 is an incretin hormone that activates the GLP-1 receptor (GLP-1R) expressed in many tissues, such as pancreas, heart, intestine, stomach, and also in white adipose tissue [9–12]. Previous studies have shown that GLP-1 or its analogs could improve mitochondrial function in pancreatic cells [13,14] but also in adipocytes [15–17]. However, whether GLP-1R signaling is involved in improvements in mitochondrial metabolism in white adipose tissue after surgery-induced weight loss remains elusive. Furthermore, the significance of GLP-1R signaling for the metabolic function of white adipose tissue is not fully understood. Thus, improving the knowledge of the metabolic regulatory role of GLP-1R signaling in white adipose tissue could facilitate the development of treatment options for white adipose tissue dysfunction in obesity.

GLP-1 analogs, such as liraglutide (LG), are used for treating type 2 diabetes and obesity [18]. They have been shown to decrease plasma glucose concentrations, induce weight loss, have cardioprotective effects, improve insulin sensitivity, and reduce low-grade inflammation in humans [19,20]. *In vivo* and *in vitro* studies have shown that LG activates GLP-1R and induces white adipose tissue browning leading to a brown adipocyte-like cell function and morphology, including increased mitochondrial biogenesis, uncoupling protein 1 (UCP-1) expression, lipolysis, energy expenditure, and thermogenesis in mice [15,16,21]. Additionally, LG has been demonstrated to decrease adipogenesis in human adipocytes [22,23] but increase adipocyte differentiation and lipid accumulation in murine adipocytes [10,24,25]. However, it is poorly understood whether LG can stimulate mitochondrial function in human adipocytes.

Since the activation of mitochondrial metabolism can protect against diet-induced obesity and related metabolic complications [1], the boosting of white adipose tissue mitochondrial activity has aroused great interest as a promising therapeutic tool against obesity. Currently, strategies to stimulate white adipose tissue mitochondria are scarce in humans [26]. Therefore, we here explored the therapeutic potential of LG on mitochondrial activity in human adipocytes. Our results demonstrate that LG boosts mitochondrial respiration and rescues cytokine-induced mitochondrial dysfunction in human adipocytes. These findings reveal a novel role for LG in the modulation of mitochondrial function in human adipocytes. Thus, one of the mechanisms underlying the beneficial effects of LG on whole-body metabolic homeostasis could be related to improved white adipose tissue mitochondrial function. Overall, our results provide new insights into the importance of GLP-1R signaling in the regulation of adipocyte metabolic health in humans.

2. Methods

2.1. Cell culture

Human Simpson Golabi Behmel syndrome (SGBS) pre-adipocyte cell strain is characterized by a high capacity for adipogenic differentiation [27], and is widely considered a representative *in vitro* model of human primary white pre-adipocytes with a lean, insulin sensitive phenotype [28]. SGBS cells were cultured as previously described with the exception that 3% FBS was included in 3FC medium during the induction of pre-adipocyte differentiation [29].

2.2. Treatments

SGBS cells were first treated with several concentrations of LG (0.001, 0.01, 0.1, and 1 μM) and corresponding vehicle (PBS) controls close to the plasma peak concentration from day 0 (pre-adipocytes) until day 15 during differentiation of adipocytes [long-term (LT-LG): D0-15] and on day 14 of post-differentiation for 24 h [short-term (ST-LG): 0–24 h] daily. LG concentration of 0.1 μM was chosen for further experiments as this dose most efficiently increased the *GLP-1R* mRNA expression, which also reflects GLP-1R activation [30]. This concentration is only slightly higher than the therapeutic plasma peak concentration of LG (up to 0.025–0.05 μM) [31]. Additionally, the effect of 0.1 μM LG during different time points of differentiation (D0-5, D0-10, and D0-15) on lipid accumulation was investigated. Moreover, differentiated adipocytes on day 14 of post-differentiation were pre-treated with 10 nM of a specific and competitive antagonist of GLP-1, exendin 9–39 (Ex9-39) and vehicle (PBS) control, for 1 h before ST-LG treatment to explore whether the effect of LG is mediated *via* the GLP-1R activation. Additionally, to investigate the ability of LG to improve inflammation-mediated mitochondrial dysfunction, differentiated adipocytes on day 14 of post-differentiation were co-treated with 10 ng/ml of cytokine, tumor necrosis factor-alpha (TNFα), and 0.1 μM of LG and corresponding vehicle (double-distilled water and PBS) control or 10 ng/ml of TNFα and vehicle (double-distilled water) control alone for 24 h.

2.3. Gene expression

Total RNA was extracted using the RNeasy mini kit (Qiagen). The RNA concentration was measured using Qubit4 Fluorometer (Thermo Scientific). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was carried out with the QuantStudio5 system (Applied Biosystems) using TaqMan™ Fast Advanced Master Mix (Applied Biosystems) or KAPA SYBR FAST qPCR Universal Master Mix (Kapa Biosystems). Cyclophilin A1 (*PPIA*) and ribosomal protein lateral stalk subunit P0 (*RPLPO*) were used as reference genes for calculating the relative expression of target genes using the formula 2^{-ddCt} in Excel [32]. The primer details are shown in Supplementary Table 1.

2.4. Protein expression

Cells were lysed in RIPA buffer (Thermo Scientific) containing freshly supplemented phosphatase and proteinase inhibitor cocktails (Thermo Scientific). The protein concentration was determined using Qubit4 Fluorometer. Equal amounts of protein (20 μg) were separated using SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare), and blocked with 5% skim milk as previously described [29]. Primary antibodies used were: anti-PORIN (1:3000, ab15895), anti-UCP-1 (1:3000, MAB6158), anti-GLP-1R (1:2000, sc-390774), and anti-GAPDH (1:5000, ab8245). Appropriate peroxidase-conjugated secondary antibodies (Pierce) were used. Signals were detected using a SuperSignal West Pico PLUS chemiluminescent detection kit (Thermo Scientific) and ImageQuant Capture-RT ECL for Windows version 1.0.1 (GE Healthcare). ImageJ version 1.45 s (ImageJ; National Institutes of Health) was used for the densitometric analysis.

2.5. Oil red O staining

Lipid accumulation was analyzed with Oil red O staining as previously described [29]. Microscopic imaging was performed with an Olympus IX70 combined with an Olympus Camedia digital camera (c-4040 zoom). Extracted Oil red O stain was quantified at a wavelength of 500 nm with a Cytation 3 microplate reader (Agilent). Data were normalized to the cells' total DNA content by using the CyQUANT Cell Proliferation Assay (Thermo Scientific). ImageJ version 1.45 s (ImageJ; National Institutes of Health) was used to quantify staining densities and lipid droplet particles. The images were converted to grayscale (8-bit) and red-stained lipid droplets were segmented (isolated) using thresholding. Next, the watershed method and particle analysis were used for the segmented figures to calculate the number and size of the lipid droplets.

2.6. Mitochondrial respiration

SGBS pre-adipocytes were seeded into the Seahorse XFe96 microplates (Agilent) at a density of 10,000 cells/well. Cells were grown to

confluence and differentiated/treated as described above. Mito Cell Stress test (Agilent) was used to explore the effect of LG on different mitochondrial respiration-related parameters. Oxygen consumption rates were obtained before and after the sequential injection of 2 μM oligomycin, 2 μM carbonyl cyanide-p-trifluoromethoxyphenylhyd razone (FCCP), and simultaneous addition of 2 μM antimycin A and 2 μM rotenone. The measurement of mitochondrial respiration *i.e.*, oxygen consumption, was recorded with XFe96 Seahorse Analyzer (Agilent), and data were normalized to the cells' total DNA content using the CyQUANT Cell Proliferation Assay (Thermo Scientific). The Bioenergetic Health Index (BHI) of the cells was calculated using the following formula: BHI = (ATP-linked × reserve capacity) / (proton leak × non-mitochondrial). Additionally, ATP-coupled respiration, proton leak and spare reverse capacity were calculated as follows; last rate measurement before Oligomycin injection - minimum rate measurement after Oligomycin injection, minimum rate measurement after Oligomycin injection – non-mitochondrial respiration, maximal respiration – basal respiration, respectively.

2.7. mtDNA analysis

The total DNA, including mitochondrial DNA (mtDNA), was extracted using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. After that, DNA concentrations were measured using a Qubit4 Fluorometer (Thermo Scientific). A standard curve with points 8, 2, 0.5, 0.1 and 0.02 ng of DNA was prepared from the pooled samples, and 2 ng of mtDNA was quantified using primers for three different mtDNA genomic regions (16S rRNA, CYTB and D-loop), while nuclear DNA was detected with primers for three nuclear DNA (ncDNA) regions (APP, HBB and B2M). The relative mtDNA amount was calculated by taking the ratio between each target mtDNA region and the geometric mean of three nuclear DNA regions (mtDNA / ncDNA). Data analysis was performed using the standard curve method with the QuantStudio5 system (Applied Biosystems). The statistical analysis was performed using the mean of three different mtDNA genomic regions relative to nuclear DNA. The primer details for mtDNA and ncDNA are shown in Supplementary Table 1.

2.8. Statistical analysis

Results were analyzed using the GraphPad Prism5 software for Windows version 5.03 (GraphPad Software, San Diego, CA), and data are provided as mean ± standard deviation (SD). Shapiro–Wilk showed a normal distribution of data and thus, parametric tests were used for the statistical analyses. Statistical significance between pre-adipocytes on day 0 and different timepoints during pre-adipocyte differentiation, as well as significance between treatments *versus* corresponding control and fold change differences between treatment groups were analyzed using one-way ANOVA with Fisher's LSD multiple comparison test or independent sample *t*-test (indicated in figure legends).

3. Results

3.1. LG induces GLP-1R expression in human SGBS adipocytes

In line with a previous study [24], we observed that the *GLP-1R* mRNA and protein were expressed both in pre-adipocytes and differentiated human SGBS adipocytes, showing the highest expression in pre-adipocytes (D0) (Supplementary Fig. 1A). The lowest *GLP-1R* mRNA expression, when compared to pre-adipocytes, was detected after five days of differentiation (-69%). After that, the *GLP-1R* mRNA expression increased gradually after 10 (-53%) and 15 days (-27%) of differentiation, as compared with D0 (Supplementary Fig. 1A). Similar expression pattern was also found for the GLP-1R protein. Although, there were no significant differences in the GLP-1R protein expression between the days of differentiation (Supplementary Fig. 1B). Thus, our findings confirm that *GLP-1R* is expressed in human adipocytes.

Next, we explored which concentration of LG activates GLP-1R the most efficiently in human SGBS cells. As GLP-1R activation increases its mRNA expression in rat *in vitro* models [30], we used the *GLP-1R* expression as a read-out for GLP-1R activation. LG at the concentration of 0.1 μM induced the highest increase in the *GLP-1R* mRNA expression in differentiated adipocytes after both ST-LG (+21%) and LT-LG (+46%) treatments (Supplementary Fig. 2A). Therefore, a LG concentration of 0.1 μM was chosen for the planned experiments. The results also verified that this LG concentration of 0.1 μM increased the protein expression of GLP-1R by +49% after LT-LG treatment (Supplementary Fig. 2B). Notably, we also confirmed that LG acts through the GLP-1R in this experimental setting by pre-treating differentiated adipocytes with Ex9-39, an antagonist for GLP-1R [24]. The results showed that Ex9-39 pre-treatment inhibited the ST-LG-induced increase in the *GLP-1R* mRNA expression (Supplementary Fig. 2C). Overall, our results demonstrate that LG can be used pharmacologically to stimulate GLP-1R activity in human adipocytes.

3.2. LT-LG induces the formation of smaller lipid droplets

First, we assessed whether LG can influence adipogenesis in human SGBS cells. The cells were treated with or without 0.1 μM LG at different time points (D0-5, D0-10, and D0-15) during differentiation. LG treatment did not change the mRNA expression of peroxisome proliferator-activated receptor gamma (*PPARγ2*), a master regulator of adipogenesis [33], when compared to the corresponding time point without LG treatment (Supplementary Fig. 3A). As expected, the *PPARγ2* mRNA expression was increased by 29- and 25-fold after 10 and 15 days of differentiation, respectively, as compared with pre-adipocytes at D0 (Supplementary Fig. 3A). Altogether, these results indicate that LG treatment does not have a major effect on adipogenesis in human adipocytes.

Next, the results demonstrated that there were no differences in lipid accumulation after LG treatment (Supplementary Fig. 3B-C). In contrast, the analysis of the lipid droplet area and the number of droplets showed that LT-LG treatment induced the formation of smaller lipid droplets (-19%) and an increase in the number of droplets (+27%) compared to the control (Fig. 1A-C). Thus, LG treatment induced the lipid droplet morphology that is typical for beige and brown adipocytes.

Since the increased release of fatty acids *via* lipolysis is a typical feature for beige and brown adipocytes [15], we investigated whether LT-LG modifies the expression of key genes involved in lipolysis. We found that LT-LG treatment up-regulated the mRNA expression of hormone-sensitive lipase (*LIPE*) by + 104% and patatin like phospholipase domain containing 2 (*PNPLA2*) by + 48% (Fig. 1D). These results imply that LG may enhance lipolysis leading to reduced adipocyte lipid droplet size.

3.3. LG treatment increases mitochondrial respiration in human adipocytes

To determine whether ST-LG and LT-LG treatments influence mitochondrial bioenergetics in human SGBS adipocytes, we measured mitochondrial respiration after ST-LG and LT-LG treatments. As shown in Fig. 2A, C, D, F, and G, basal respiration (+17%), maximal respiration (+24%), ATP production (+23%) and spare respiratory capacity (+29%) were increased after ST-LG treatment. Interestingly, pre-treatment with Ex9-39 attenuated ST-LG-induced effects in the above-mentioned parameters (Fig. 2C, D, F, and G) suggesting that LG-induced effects on mitochondrial respiration were mediated through the GLP-1R. After LT-LG treatment, basal respiration (+57%), maximal respiration (+71%), proton leak (+59%), ATP production (+56%), spare respiratory capacity (+82%) and non-mitochondrial respiration (+73%) were increased (Fig. 2B, C–H). In contrast, the BHI index, a single value for the bioenergetic health calculated from previous

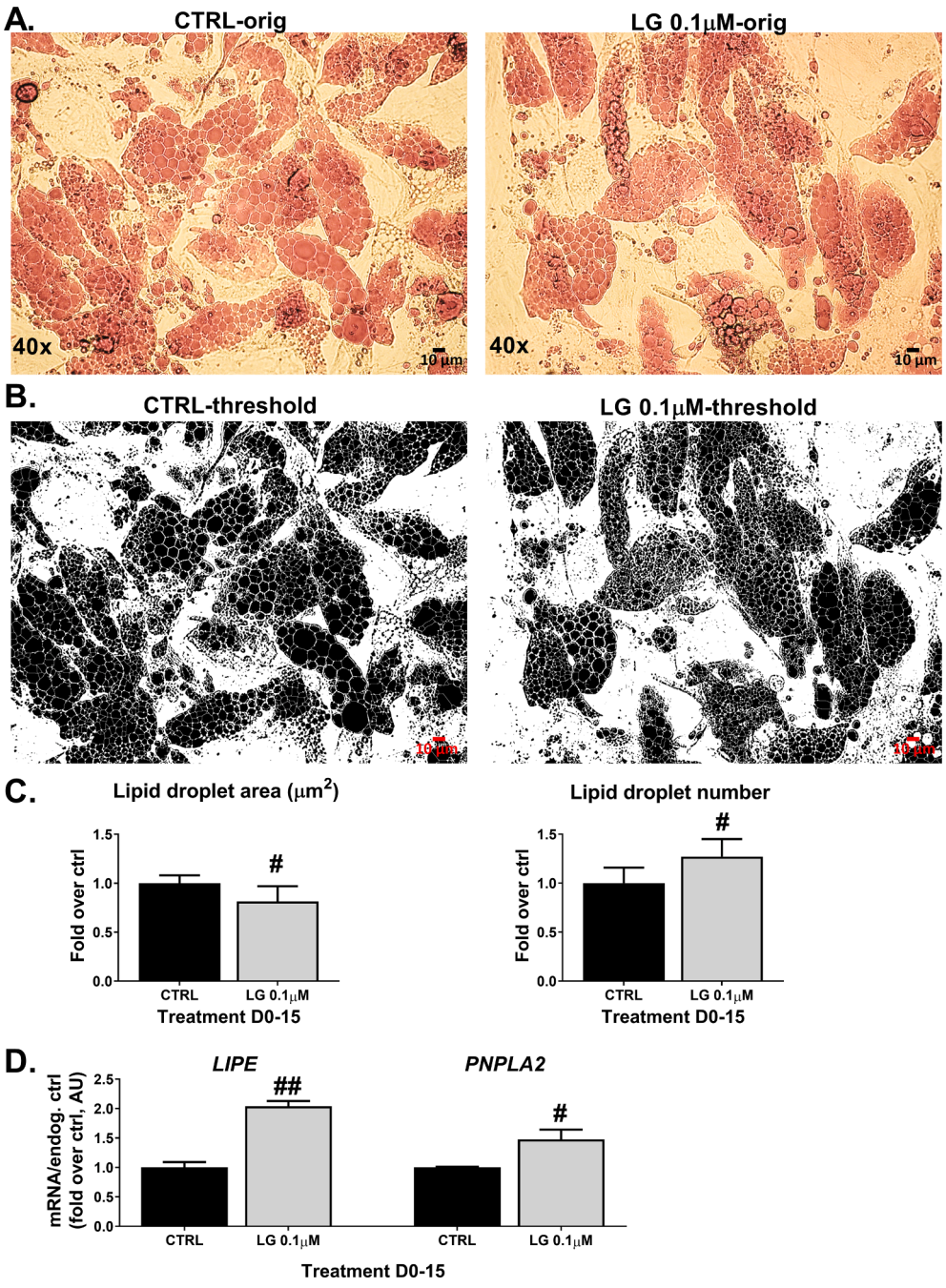

Fig. 1. The effect of LG on lipid droplet morphology and markers of lipolysis. **A)** Representative original (orig) microscopic bright field images comparing the effect of LT-LG treatment on the lipid droplet accumulation stained with Oil red O (ORO), (40x magnification, scale bar 10 μm). **B)** Positive staining of lipid droplets was chosen with thresholding and watershed algorithm was used for the lipid droplet particle analysis. Particle analysis quantification of **C)** lipid droplet area as size (μm^2) and lipid droplet number. **D)** The effect of LT-LG treatment on *LIPE* and *PNPLA2* mRNA expression. Altogether 3 bright field images/technical replicates were taken. Values are presented as means ± SD, n = 3 independent biological replicates, including 3 technical replicates/biological replicate. Statistics: independent samples *t*-test, # $p < 0.05$, ## $p < 0.01$ (C, D: compared to corresponding control). D: day, LT-LG: long-term (D0-15), CTRL: control, LG: liraglutide, LIPE: hormone-sensitive lipase, PNPLA2: patatin like phospholipase domain containing 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parameters, did not differ between control and ST-LG or LG-treated adipocytes (Supplementary Fig. 4). These results demonstrate that both ST-LG and LT-LG treatments promote adipocyte mitochondrial respiration potentially through GLP-1R. Additionally, long-term exposure to LG during the differentiation, compared with short-term LG treatment in differentiated adipocytes, has a more profound effect on mitochondrial respiratory function in human adipocytes.

3.4. LT-LG induces browning and mitochondrial biogenesis

As the browning of adipocytes could be causing the observed changes in adipocyte morphology and mitochondrial respiration, we investigated whether LG stimulates this process in human adipocytes as in mice [15,34]. Of the browning markers, ST-LG treatment increased the mRNA expression of the key markers for beige and brown adipocytes, *UCP-1,* and *CIDEA* by +69% ($p = 0.003$) and by +66% ($p = 0.021$), respectively (Supplementary Fig. 5A). As shown in Fig. 3A-B, the mRNA and protein expression of *UCP-1* was increased by +86% and +114%, respectively, after LT-LG treatment. Additionally, LT-LG treatment up-regulated mRNA expression of other beiging/browning markers, such as PPARG Coactivator 1 Alpha (*PGC1α*) by +100%, PR domain-containing 16 (*PRDM16*) by +35%, cell death-inducing DFFA-like effector A (*CIDEA*) by +117% and transmembrane protein 26 (*TMEM26*) by +71% (Fig. 3A). Taken together, these results indicate that LG induces the browning of human white adipocytes.

Given that browning is typically associated with increased mitochondrial biogenesis, we also determined whether LG increased the commonly used read-outs for mitochondrial number and mass. ST-LG treatment increased mtDNA amount by +44% and the protein expression of a mitochondrial mass marker, PORIN/VDAC, by +26%

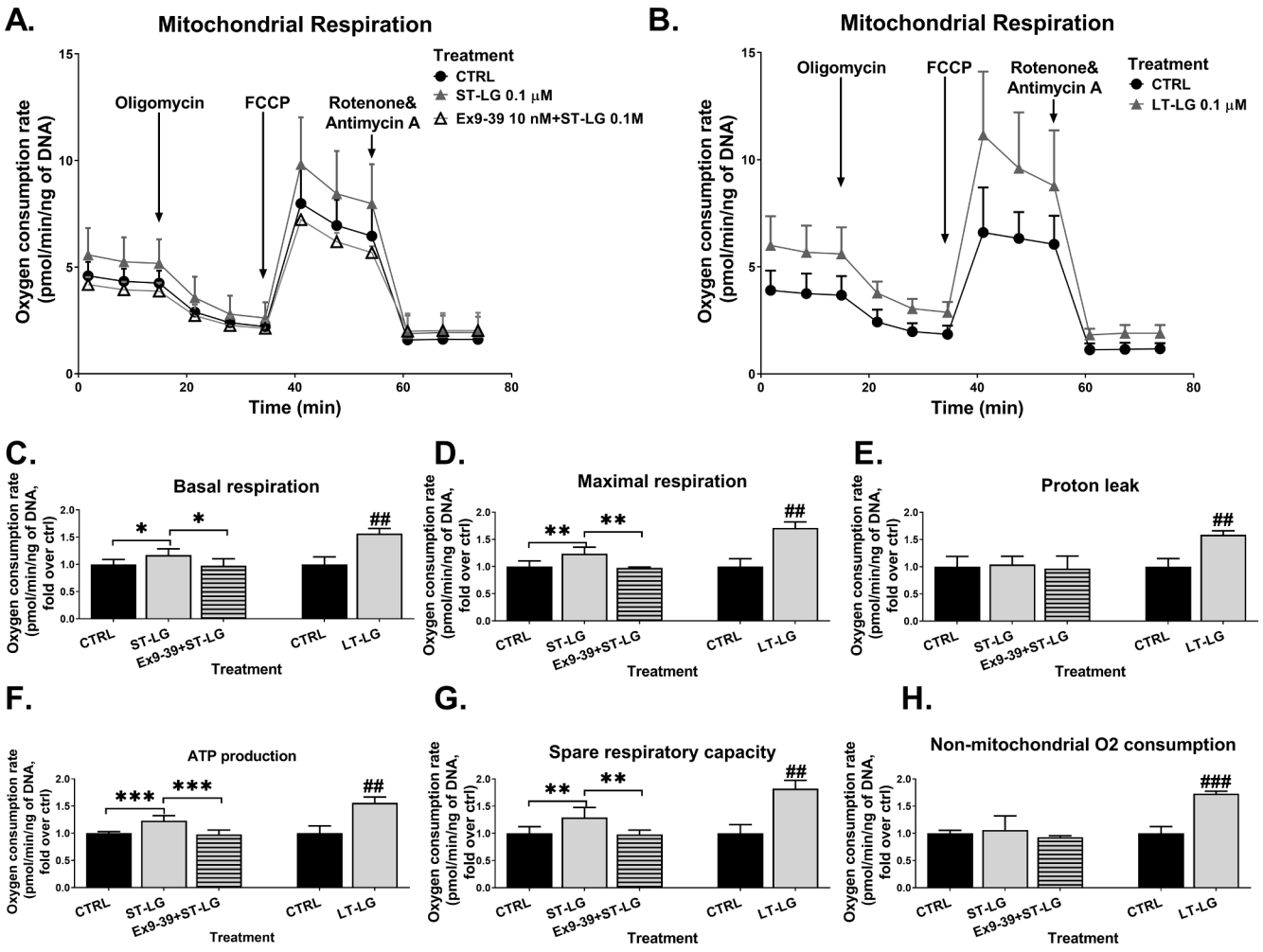

Fig. 2. The effect of LG on mitochondrial respiration. **A, B)** Schematic representation of mitochondrial respiration curves obtained from different treatments. The effect of ST-LG treatment with and without 10 nM Ex9-39 pre-treatment (1 h), and LT-LG treatment on mitochondrial respiration, presented as the parameters of mitochondrial respiration **C)** basal respiration, **D)** maximal respiration, **E)** proton leak, **F)** ATP production, **G)** spare respiratory capacity, and **H)** non-mitochondrial respiration. Values are presented as means ± SD, n = 3–6 independent biological replicates, including 8 technical replicates/biological replicate. Statistics: ST-LG, one-way ANOVA with Fisher's LSD multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); LT-LG, independent samples *t*-test (## $p < 0.01$, ### $p < 0.001$). ST-LG: short-term (24 h), Ex9-39: exendin9-39, LT-LG: long-term (D0-15), CTRL: control, LG: liraglutide, D: day.

(Supplementary Fig. 5B, C). mtDNA amount (Fig. 3C) increased by +24% and the protein expression of PORIN/VDAC (Fig. 3B) was significantly elevated by +68% after LT-LG treatment. Therefore, these results demonstrate that LG promotes mitochondrial biogenesis likely resulting in increased mitochondrial content in human adipocytes.

3.5. ST-LG improves cytokine-induced defects in mitochondrial respiration

Next, we explored whether LG treatment improves cytokine-induced mitochondrial dysfunction typically occurring in adipocytes in obesity [2]. As we have previously established a SGBS cell model with pro-inflammatory cytokine, TNFα, -induced insulin resistance [35], we investigated whether mitochondrial dysfunction was induced after TNFα treatment in these human adipocytes. In this model, maximal respiration (–22%), spare respiratory capacity (–33%), and BHI (-39%) (Fig. 4C, F, and H) were reduced after TNFα treatment. Importantly, the ST-LG treatment reversed the TNFα-induced mitochondrial bioenergetic defects as the co-treatment with TNFα and LG restored maximal respiration, spare respiratory capacity, and BHI to the same level as the control group (Fig. 4C, F and H). This finding revealed that LG can restore cytokine-induced mitochondrial dysfunction in the metabolically unhealthy state in human adipocytes.

3.6. LG ameliorates cytokine-induced effects on inflammation and mitochondrial biogenesis

Next, we investigated the underlying reasons for improved mitochondrial respiration after the co-treatment with TNFα and ST-LG. First, we determined the effect of TNFα treatment on inflammation in our SGBS cell model. As expected, our results showed an increase in the mRNA expression of *TNFα* by +97% and interleukin 1 beta (*IL1β*) by +43% after TNFα treatment (Fig. 5A). The co-treatment with ST-LG significantly reduced the expression of these TNFα-induced increases in mRNA expression (by −97% for *TNFα* and −38% for *IL1β*) (Fig. 5A). TNFα treatment increased the mRNA expression of the inhibitor of nuclear factor kappa B kinase subunit beta (*IKKB*) by +43% (Fig. 5A), the activator of NF-κB signaling [36] while nuclear factor kappa B inhibitor alpha (*IKBα*), the inhibitor of the same signaling pathway [36], remained unchanged (Fig. 5A). In contrast, ST-LG normalized *IKKB* mRNA expression and significantly increased *IKBα* expression by +57% compared with TNFα alone (Fig. 5A). Thus, these results indicate that the positive effect of LG on mitochondrial respiration may be at least partly mediated via attenuated inflammation upon the co-treatment of TNFα with ST-LG.

Secondly, we analyzed sirtuin (*SIRT)1* mRNA expression, the mediator of LG's inhibitory effect on inflammation *via* NF-κB signaling

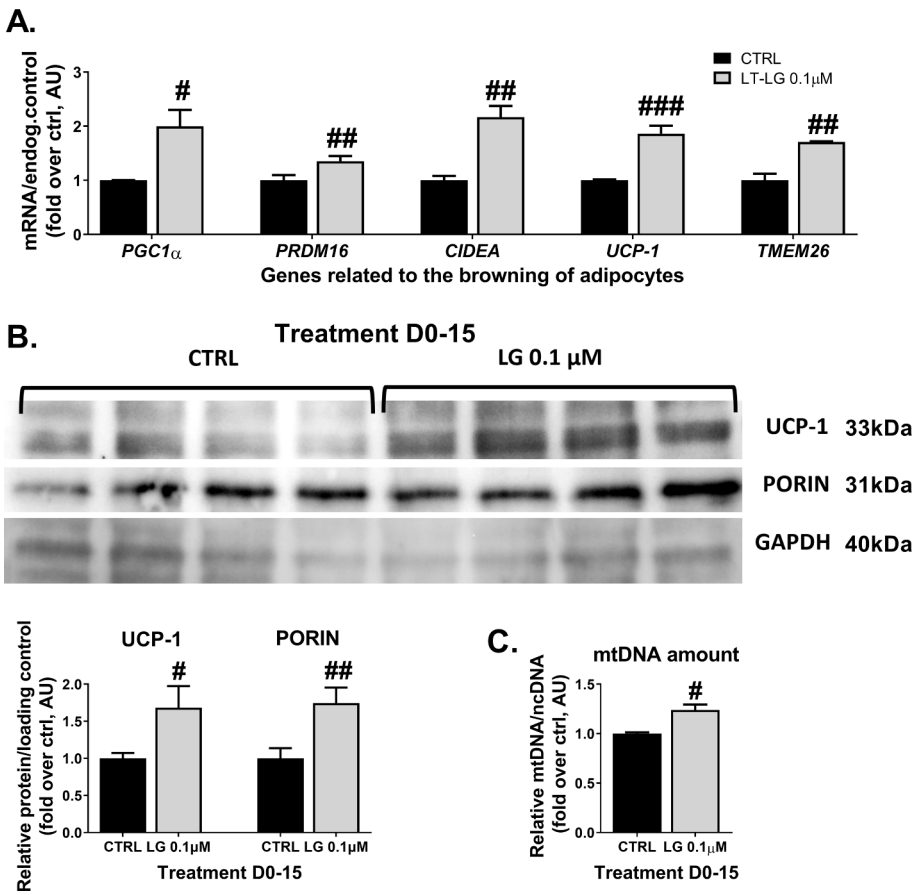

Fig. 3. The effect of LG on the browning of adipocytes and mitochondrial biogenesis. The effect of LT-LG treatment **A)** on the expression of genes related to adipocyte browning, **B)** on the protein expression of UCP-1 and PORIN using densitometric analysis, and **C)** on the mitochondrial DNA (mtDNA) amount. Values are presented as means ± SD, n = 3 independent biological replicates, including 3 technical replicates/biological replicate. Statistics: independent samples *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to corresponding control). D: day, LT-LG: long-term (D0-15), CTRL: control, LG: liraglutide.

[34,37]. *SIRT1* mRNA expression was reduced by −12% after TNFα treatment and increased by +34% after co-treatment with ST-LG compared with TNFα alone (Fig. 5B). Finally, we investigated the effects of TNFα and ST-LG on adipocyte browning and mitochondrial biogenesis in the co-treatment model. TNFα treatment did not affect the mRNA or protein expression of *UCP-1*, but after the co-treatment with TNFα and ST-LG, both *UCP-1* mRNA (+42%) and protein (+16%) expression levels were significantly higher compared with TNFα alone (Fig. 5B-D). Both the mRNA expression of *PGC1α* and the protein expression of PORIN/VDAC were significantly higher after co-treatment with ST-LG by + 52% and 44%, respectively, when compared with TNFα alone (Fig. 5B-D). Altogether, these results demonstrate that browning and improved mitochondrial biogenesis likely play a role in the LG-induced beneficial effects on mitochondrial respiration upon inflammation in human adipocytes.

4. Discussion

Currently, there are no pharmacological approaches available for the activation of mitochondria for clinical use in obesity and related metabolic complications [2,38]. The anti-obesity and anti-diabetic compound, LG, has recently emerged as a potential regulator of mitochondrial function based on mouse studies [15,16,21]. Here we show that both ST-LG and LT-LG treatments promoted mitochondrial respiration, likely through GLP-1R, in human SGBS adipocytes. Importantly, we demonstrated that ST-LG could rescue TNFα-induced defects in mitochondrial metabolism and inflammation. Therefore, LG demonstrates a therapeutic potential against inflammation-induced mitochondrial dysfunction in human adipocytes.

Previous studies have shown that the target of LG, GLP-1R, is expressed in human and mouse white adipose tissue and *in vitro* human- and murine-derived pre-adipocytes and differentiated adipocytes [9,10,12,24]. Additionally, LG has been shown to activate GLP-1R in mouse adipocytes [10,24]. In line with these previous studies, we confirmed the *GLP-1R* mRNA expression in human SGBS pre-adipocytes and differentiated adipocytes. Furthermore, both ST-LG and LT-LG treatments induced an increase in the *GLP-1R* mRNA expression, thereby suggesting an increased GLP-1R activation in human adipocytes. Importantly, we confirmed using the antagonist for GLP-1R, Ex9-39, that the effect of LG on *GLP-1R* expression depends on GLP-1R signaling in human SGBS adipocytes. Our findings demonstrate for the first time that LG stimulates GLP-1R activity in human adipocytes.

The activation of GLP-1R leads to increased insulin sensitivity and reduced inflammation in non-adipose tissues [15]. Interestingly, GLP-1R analog, LG, has been suggested to induce mitochondrial function in white adipose tissue and adipocytes in mice [15,16,21] but the effect of LG in human adipocytes has remained elusive. Here, we provide novel evidence that ST-LG and LT-LG treatments stimulate mitochondrial respiratory function in human SGBS adipocytes. Elevated maximal respiration and spare respiratory capacity were observed after both ST-LG and LT-LG treatment. This indicates that LG increases mitochondrial oxidative capacity and enables adipocytes to better adjust their ATP production rate to the current cellular energy status. Notably, the observed beneficial changes in mitochondrial respiration were mediated *via* GLP-1R as the antagonist for GLP-1R, Ex9-39, blocked the effect of ST-LG treatment on mitochondrial respiration in SGBS adipocytes. Thus, our findings highlight the GLP-1R-dependent effects of LG on mitochondrial function in human adipocytes and underscore that the GLP-1R signaling plays a role in the regulation of adipocyte energy metabolism.

LG triggers white adipose tissue browning and mitochondrial biogenesis based on *in vivo* and *in vitro* studies in mice [15,16,21]. Here we demonstrate for the first time in human adipocytes that mRNA and

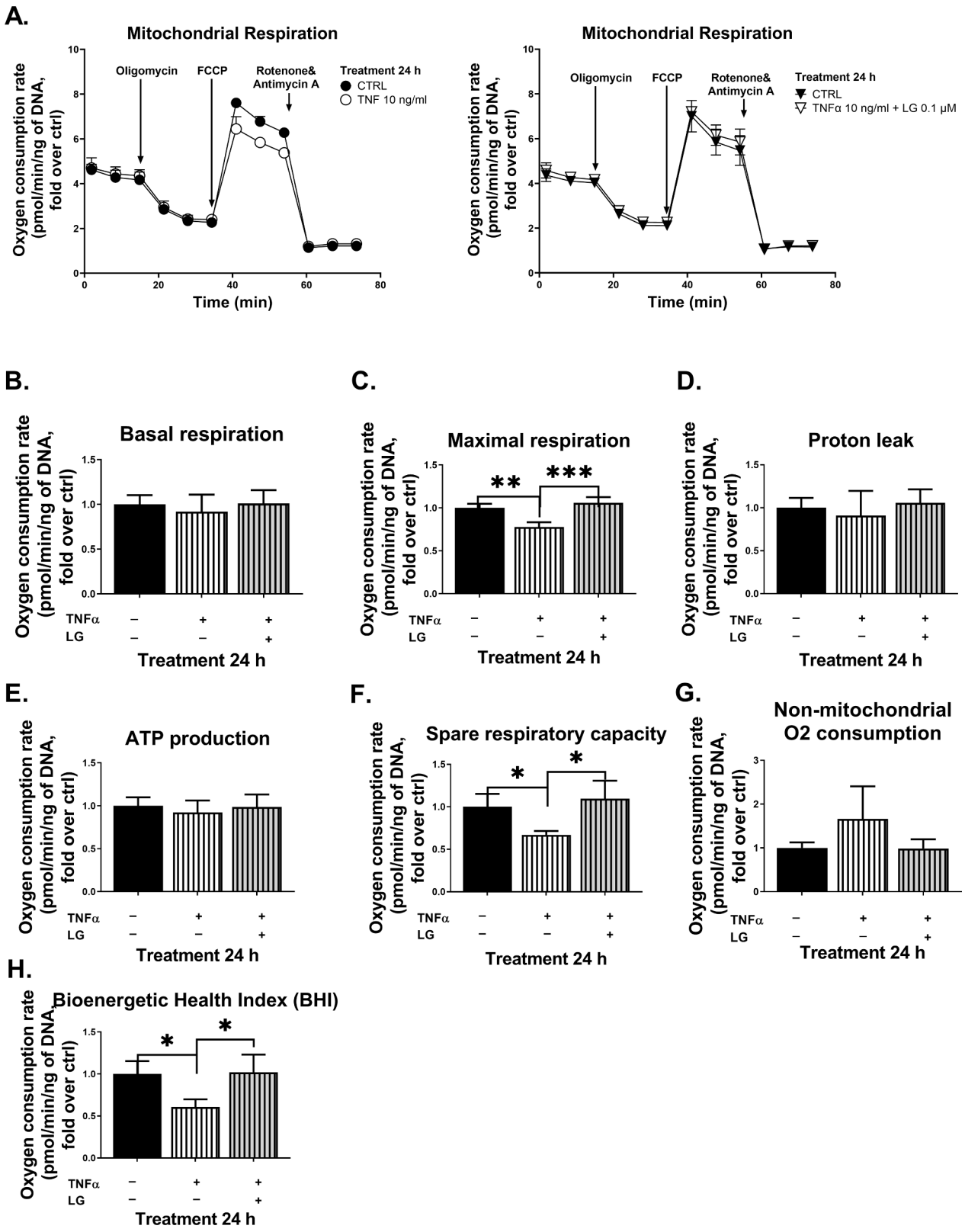

Fig. 4. The effect of LG on tumor necrosis factor-alpha (TNFα) -induced mitochondrial bioenergetic defects. **A)** Schematic representation of mitochondrial respiration curves obtained from different treatments. The effect of TNFα (10 ng/ml) and ST-LG (0.1 μM) co-treatment (24 h) on mitochondrial respiration presented as the parameters of mitochondrial respiration **B)** basal respiration, **C)** maximal respiration, **D)** proton leak, **E)** ATP production, **F)** spare respiratory capacity, **G)** non-mitochondrial respiration and **H)** bioenergetic health index (BHI). Values are presented as means ± SD, n = 3 independent biological replicates, including 8 technical replicates/biological replicate. Statistics: one-way ANOVA with Fisher's LSD multiple comparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to corresponding control). ST-LG: short-term (24 h), CTRL: control, LG: liraglutide.

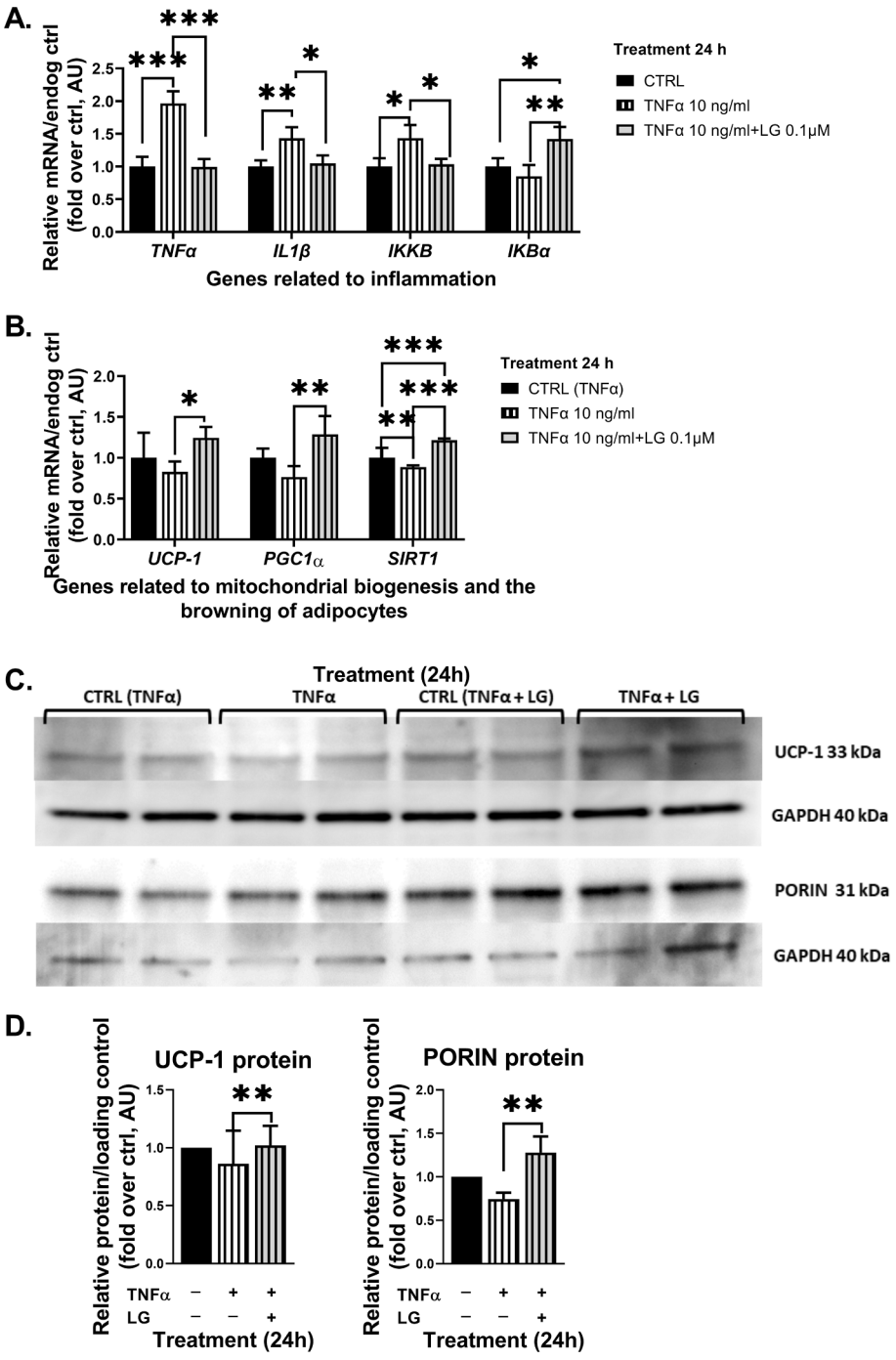

Fig. 5. The effect of LG on TNFα -induced inflammation and browning of adipocytes. The effect of TNFα (10 ng/ml) and ST-LG (0.1 μM) co-treatment (24 h) and 10 ng/ml of TNFα or vehicle for 24 h **A)** on the expression of pro-inflammatory genes, **B)** on the expression of genes related to mitochondrial biogenesis and adipocyte browning and **C, D)** on the protein expression of UCP-1 and PORIN. Values are presented as means ± SD, n = 3 independent biological replicates, including 3 technical replicates/biological replicate. Statistics: one-way ANOVA with Fisher's LSD multiple comparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to corresponding control). ST-LG: short-term (24 h), CTRL: control, LG: liraglutide, TNFα: tumor necrosis factor-alpha.

protein levels of UCP-1, a specific marker of brown adipose tissue [15], were significantly elevated in SGBS adipocytes after LG treatment. Additionally, LT-LG up-regulated mRNA expression of other beige/brown adipose tissue markers, including *PGC1α* [15], *PRDM16* [39], *TMEM26* [40], and *CIDEA* [41] in human SGBS adipocytes. LT-LG treatment also elevated mtDNA amount and increased the protein expression of the mitochondrial mass marker, PORIN/VDAC, in SGBS adipocytes. Therefore, LT-LG mediated increase in mitochondrial respiration, at least partially, resulted from the induction of browning and increased mitochondrial number in SGBS adipocytes.

LG has been suggested to influence adipogenesis in both mouse and human adipocytes [10,22–25]. In this study, LT-LG did not affect adipocyte differentiation as the mRNA expression of *PPARγ2*, the master regulator of adipocyte differentiation [33], remained unchanged. This finding excluded the possibility that increased adipogenesis, that typically aligns with increased mitochondrial metabolism [1], was the key mediator of increased mitochondrial respiratory function upon LG. Interestingly, LT-LG induced an increase in the number of smaller lipid droplets and the upregulation of *PNPLA2* (ATGL) and *LIPE* (HSL), the key enzymes involved in the degradation of triacylglycerol (lipolysis). Previous study has shown that increased lipolysis in murine adipocytes leads to decreased lipid droplet size [42]. Thus, our findings indicate that LG could stimulate lipolysis resulting in smaller adipocyte lipid droplet size. This notion is supported by previous results in mice, demonstrating the reduced size of white adipocytes and an increased lipolytic activity upon LG treatment [15,43]. Altogether, our findings revealed that LT-LG influences the degradation of triacylglycerols rather than adipogenesis in human SGBS adipocytes.

White adipose tissue low-grade inflammation is typically associated with mitochondrial dysfunction in obesity [44]. Here we established a SGBS cell model with a pro-inflammatory cytokine (TNFα)-induced mitochondrial dysfunction to mimic the mitochondrial bioenergetics defects typically observed in white adipose tissue in obesity. Previous evidence suggests that LG has anti-inflammatory effects that include the suppression of NF-κB signaling and the expression of pro-inflammatory genes [37,45–47]. Importantly, we found for the first time that ST-LG could reverse TNFα-induced impairment in mitochondrial respiration in human adipocytes. In agreement with previous *in vitro* studies [46,47], the underlying mechanism was most likely related to the anti-inflammatory effect of LG and increased browning and mitochondrial biogenesis. Overall, our findings imply that LG could improve mitochondrial dysfunction in adipocytes in the metabolically unhealthy state. Therefore, the well-tolerated anti-obesity drug, LG, could be used pharmacologically to target adipocyte mitochondria in obese individuals. This is of importance since there is a lack of good bioactive molecules without side effects to stimulate mitochondrial metabolism in humans [2,38]. Overall, our results provide new mechanistic insight that the LG-mediated beneficial effects on whole-body metabolic homeostasis could be partially linked to increased mitochondrial activity in white adipose tissue.

The limitation of this study was that we investigated the effect of LG specifically on adipocytes and not on all cell types present in white adipose tissue. Thus, it would be important in the future to use white adipose tissue organoids, which mimic more closely the *in vivo* white adipose tissue [48]. To explore the possible treatment effects of LG on obesity associated metabolic disturbances, these organoids could be subjected to LG in metabolic conditions typically occurring in white adipose tissue in obesity. Additionally, adipocytes from distinct anatomical locations [49], and between an early age and in adulthood have different gene expression profile and functional properties [50]. The experiments were conducted only with one type of adipogenic cell strain (SGBS) which has a large beige potential [50]. Further experiments are needed to unravel what effects liraglutide exert on adipocyte subpopulations that are brown prone (e.g. neck adipocytes) or have limited browning capacity (e.g. omental adipocytes).

4.1. Conclusions

In conclusion, LG promotes mitochondrial respiration and metabolic flexibility, as well as induces mitochondrial biogenesis in human adipocytes, possibly through an increased brown-like phenotype. Notably, our data suggest that LG could reverse cytokine-induced adipocyte mitochondrial dysfunction. Therefore, our evidence suggests that LG could be a mitochondrial activator of human adipocytes and thus, contribute to improved metabolism in individuals with obesity.

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CRediT authorship contribution statement

Maija Vaittinen: Methodology, Investigation, Formal analysis, Writing – original draft, Project administration, Visualization. **Mariana Ilha:** Investigation, Writing – review & editing. **Elena Herbers:** Resources, Writing – review & editing. **Anita Wagner:** Resources, Writing – review & editing. **Kirsi A. Virtanen:** Supervision, Writing – review & editing. **Kirsi H. Pietiläinen:** Conceptualization, Resources, Writing – review & editing. **Eija Pirinen:** Formal analysis, Conceptualization, Resources, Writing – review & editing. **Jussi Pihlajamäki:** Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2023.110635.

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