Glucagon-like peptide-1 receptor agonist stimulates mitochondrial bioenergetics in human adipocytes*

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Glucagon-like peptide 1 receptor agonists (GLP-1RAs) are relatively new pharmacological agents used to normalized glucose level in type 2 diabetes. Recently, GLP-1RAs have been approved for the treatment of obesity to reduce body weight in non-diabetic patients. The extra-pancreatic effects of GLP-1RAs, as well as their molecular mechanism of action, are still poorly understood. Thus this study was aimed to verify the hypothesis that the mechanism of action of the GLP-1RAs involves mitochondria and that GLP-1RAs administration can improve mitochondrial functions. For this purpose, preadipocytes CHUBS7 were differentiated to mature adipocytes and then stimulated with GLP-1RA, exendin-4 at 100 nM for 24 h. Oxygen consumption rates, mitochondrial membrane potential, intracellular ATP (adenosine triphosphate) level, SIRT1 and SIRT3 gene expression and the histone deacetylases’ activity were measured. Exendin-4 was found to uncouple mitochondrial electron transport from ATP synthesis, slightly decreasing mitochondrial membrane potential in mature adipocytes. Routine respiration and uncoupled oxygen consumption rates were higher in exendin-4 treated adipocytes than in the non-treated cells. The ATP level remained unchanged. Exendin-4 enhanced SIRT1 and SIRT3 genes expression. Histone deacetylases’ activity in the nuclear fraction was not affected by exendin-4, although the activity of class III histone deacetylases was increased. All of the effects on mitochondrial bioenergetics induced by exendin-4 were abolished by addition of glucagon-like peptide 1 receptor antagonist. In conclusion, exendin-4 activates the sirtuin pathway and increases energy expenditure in human adipocytes. Our results suggest another mechanism that may be responsible for body weight reduction observed in patients using GLP-1RAs.

Key words: exendin-4, GLP-1, mitochondria, sirtuin, mitochondrial respiration

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Abbreviations: ATP, adenosine triphosphate; BAT, brown adipose tissue; Ex-4, exendin-4; ETC, electron transport chain; GLP-1, Glucagon-like peptide 1; GLP-1RA, Glucagon-like peptide 1 receptor agonist; HDAC, histone deacetylases; OCR, oxygen consumption rate; sirt, sirtuin 1; sirt3, sirtuin 3; WAT, white adipose tissue

INTRODUCTION

Mitochondria are at the center of cellular metabolism and not only provide energy for a variety of metabolic processes but also are central for the coordination and integration of the various pathways defining the cell’s metabolic program. It is well established that mitochondria are critically involved in the pathophysiology of metabolic disorders, such as obesity and diabetes (Muozio & Newgard, 2008). Mitochondrial function also appears to be a critical determinant of insulin sensitivity within the muscle, liver, and adipose tissue (Szendroedi et al., 2011).

Incretins, gut-derived hormones, include glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic peptide (GIP), that are secreted into the circulation at low basal levels at a fasting state but increasing rapidly and transiently following food ingestion. GLP-1 is released in response to food, especially glucose, fatty acids, proteins and bile salts in the bowel. The biological effects of GLP-1 principally include stimulation of insulin expression and insulin biosynthesis (Fehmann et al., 1997). GLP-1 has been also shown to prevent β-cell apoptosis in animal models of diabetes (Li et al., 2003) and glucolipotoxicity (Buteau et al., 2004). The GLP-1 plasma level is decreased and consecutively the insulinotropic effect of this incretin is diminished in type 2 diabetes, in obesity or after bariatric surgery (Faehr et al., 2015).

GLP-1 is rapidly degraded in vivo by dipeptidyl peptidase IV (DPP IV) located in various tissues, on the capillary surfaces, as well as present in a soluble form in the circulation, so active GLP-1 (7-36) has a half-life of 2 min. Short half-life of native GLP-1 has been overcome through the use of chemically modified degradation-resistant glucagon-like peptide 1 receptor agonists (GLP-1RAs) in therapy of type 2 diabetes. GLP-1RAs mimic the effects of native GLP-1, which increases insulin secretion, inhibits glucagon secretion, increases satiety and slows gastric emptying (Ostergaard et al., 2016). The most often used GLP-1 receptor agonists, exenatide and liraglutide, administered together with oral antihyperglycemic agents have been shown to be effective in the treatment of type 2 diabetes. Exenatide (exendin-4) is a synthetic agonist of the GLP-1 receptor, approved (in 2005 in the U.S., in 2006 in the E.U.) for the treatment of type 2 diabetes (Drucker et al., 2010). Other therapeutic GLP-1RAs, exhibiting a more prolonged duration of action, include liraglutide (approved in the E.U. in 2009) and exenatide (approved in the E.U. in 2013) and albiglutide (approved in the E.U. in 2014). GLP-1R agonists control blood glucose through stimulation of insulin and inhibition of glucagon secretion (Drucker, 2006). These GLP-1R-related effects are plasma glucose dependent, thereby minimizing the risk of hypoglycemia in the absence of concomitant sulfonylurea therapy (Butler et al., 2010; Feher et al., 2017).
In addition to glycemic control, the observed effect of GLP-1RAs is the reduction in body weight in the majority of treated subjects (Wadden et al., 2013; Lundkvist et al., 2017; le Roux et al., 2017). This well-documented effect results partially from reduced appetite (through the action on GLP-1 receptors in the hypothalamus) and slowed gastric emptying (McAdam-Marx et al., 2016). Moreover, an ultrasound study documented that even a short course of treatment with exenatide, besides weight loss, induces a redistribution of adipose tissue deposits, possibly contributing to a better cardiovascular risk profile in patients with type 2 diabetes (Morano et al., 2015). After successful trials with GLP-1RAs at a high dose in non-diabetic subjects, the incretin mimetic therapy has been approved (in the U.S. in 2014, and in March 2015 in the E.U.) for treatment of obesity to reduce body weight in obese non-diabetic patients (Anandhakrishnan & Korbonits, 2016).

Due to the growing interest in incretin therapy, several clinical as well as experimental studies are in progress, although the mechanism of GLP-1RAs action in adipose tissue is not yet sufficiently understood (Majumdar & Weber, 2010; Chae et al., 2015; Pastel et al., 2016). Thus, our study was aimed to verify the hypothesis that the GLP-1 RA mechanism of action involves mitochondria and that GLP-1RA administration can improve mitochondrial functions. For this purpose, the influence of GLP-1 RA, namely exendin-4, on mitochondrial bioenergetics was studied in the CHUBS7 human adipocytes.

**MATERIALS AND METHODS**

**Cell culture.** All reagents were purchased from Sigma-Aldrich, unless stated otherwise. Human cell line CHUBS7 (Nestle Research Center, Lausanne, Switzerland) derived from human subcutaneous adipose tissue, was cultured in a mixture (1:1) of DMEM and F12 culture media. Cells were plated (at a density of 30×10⁴ cells/cm²) in DMEM/F12 media supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA). At confluence, the cells were incubated in a basal medium, which was serum-free DMEM/F12 medium supplemented with 15 mM NaHCO₃, 17 μM D-pantethanic acid, 15 mM Hepes and 33 μM biontin, 10 μg/ml transferrin, 1 nM triiodothyronine, 850 nM insulin, and freshly added 500 μg/ml feticin. The CHUBS7 cells were differentiated into mature adipocytes by adding 1 μM dexamethasone and 1 μM rosiglitazone to the basal medium for 21 days. Then adipocytes were exposed to GLP-1 agonist, exendin-4 (Ex-4) (100 nM) or to Ex-4 along with GLP-1 antagonist, exendin-9 (Ex-9) (100 nM), for 24 hours.

**Cell differentiation assay.** The cellular lipid content was assessed by lipid staining with Oil Red-O. Cells were washed twice with PBS and fixed in 3.7% paraformaldehyde (POCh, Gliwice, Poland) in PBS without Mg²⁺ and Ca²⁺ (Biomed, Lublin, Poland) for 1 hour at room temperature. Then, the paraformaldehyde solution was removed, cells were washed shortly with double distilled H₂O and stained with Oil Red-O solution for 1 hour at room temperature. For the staining, 0.5 g of Oil Red-O was dissolved in 100 ml of isopropanol (POCh, Gliwice, Poland). After 1 hour, the Oil Red-O containing medium was removed and the cells were examined by light microscopy.

**Oxygen consumption.** Mitochondrial respiration assay was performed using the high-resolution respirometry system Oxygraph-2k (Oroboros Instruments, Austria). Live CHUBS7 cells (1×10⁴) were suspended in 2 ml of basal medium containing 5 mM glucose (control cells) or in 2 ml of basal medium containing 5 mM glucose with exendin-4 (Ex-4), exendin-9 (Ex-9) or in a medium containing 25 mM glucose (high glucose control). Oxygen sensors were calibrated routinely at air saturation and in oxygen depleted media. Oxygen consumption was measured at 37°C in a thermostatically controlled chamber. The protocol according to Dunham-Snary and coworkers (2014) was used.

**Intracellular ATP content.** The intracellular ATP content was measured by using the ATPLiteTM luminescence assay (Perkin Elmer, Waltham, MA, USA), according to the protocol provided by the manufacturer, and the luminescence was measured using a Tecan Genios microplate reader (Thermo Fisher). Data were calculated using the Magellan software. Protein content was measured in the samples of cell lysates by Total Protein Kit, Micro Lowry, Peterson’s Modification (Sigma). The ATP concentration was adjusted for protein content in each sample and expressed as nmol ATP/mg of cell protein.

**Mitochondrial membrane potential (ΔΨₘ).** Changes of the ΔΨₘ were monitored by fluorescent staining of the live CHUBS7 cells with JC-1 dye. The cells were incubated with 2 μM JC-1 solution (MitoProbe Assay Kit, Invitrogen Life Technologies, Carlsbad, CA, USA) in the dark for 45 min at 37°C. Then, the cells were washed, diluted in PBS and analyzed by flow-cytometry (FACS-Canto II, BD Biosciences Discovery Labware, Bedford, MA, USA) using 488 nm excitation with 530/30 nm (FL1, green) and 585/42 nm (FL2, orange) band pass emission filters. Fluorescence of 1×10⁶ cells was collected during a single instrument run. The data were analyzed using the FacsDIVA software (BD Biosciences Discovery Labware, Bedford, MA, USA). In live cells, JC-1 exists either as a green-fluorescent monomer at depolarized membrane potentials or as an orange-fluorescent J-aggregate at hyperpolarized membrane potentials. The mitochondrial membrane potential was quantified by a ratio of red to green fluorescence intensities generated by JC-1 staining. This ratio is dependent only on the ΔΨₘ and not on other factors such as mitochondrial size, shape, and density.

**Relative gene expression.** Total RNA was isolated from cells using a TRIZOL® Plus RNA Purification System (Life Technologies, Carlsbad, CA, USA). The quality of RNA was confirmed by analysis on the NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA). One microgram of total RNA was reverse transcribed using a reverse transcription kit (High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) with random primers. Subsequently, cDNA was subjected to real-time PCR. Quantitative real-time polymerase chain reaction (qPCR) was performed with the TaqMan® Gene Expression Assays, according to the manufacturer’s protocol using TaqMan® primers for SIRT1 (Hs01009006_m1) and SIRT3 (Hs00953477_m1) (Applied Biosystems). As a reference gene, expression of 18S rRNA was used (Hs03003631_g1) (Applied Biosystems). Amplification was performed using the continuous fluorescence detection system 7900 HT Fast Real Time PCR system (Applied Biosystems). Data were obtained in a form of sigmoid amplification plots in which fluorescence was plotted against the number of cycles. The threshold cycle (CT) served as a measurement of the starting template amount in each sample. Expression ratio was calculated as normalized CT difference between the control probe and the sample.
Histone deacetylases activity. Nuclear fraction was extracted from CHUBS7 cells using Nuclear Extraction Kit according to the protocol supplied by the manufacturer (Merck Millipore, Darmstadt, Germany). The protein concentration was determined by Total Protein Kit, Micro Lowry, Peterson’s Modification (Sigma). Each nuclear extract sample (10 µg) was pipetted into four microplate wells, and was run in duplicates with and without addition of trichostatin A (TSA). Histone deacetylases’ activity was detected using colorimetric HDAC Assay Kit (Millipore), according to the protocol supplied by the manufacturer. Briefly, samples were incubated with HDAC assay substrate for 60 minutes at 37°C, allowing deacylation of the substrate. Next, the activator solution, incubated for 20 minutes at RT, released the p-nitroanilide from deacylated substrate. Colorimetric p-nitroanilide was measured in a plate reader (Multiscan) at 405 nm. The HDAC activity was calculated using a standard curve prepared with an assay standard and normalized to protein content.

Statistical analysis. All of data were expressed as the mean ±S.D. from five independent experiments measured in triplicate. Calculation of respective gene expression was performed using the comparative CT method. Statistical significances for comparisons between treated and control cell samples were determined by the unpaired t-test using Statistica 10.0 PL software.

RESULTS

Cell differentiation

The morphology of cultured CHUBS7 cells and lipid droplets formation were observed with the light microscopy. After 21 days of culture, the cells were stained with Oil Red-O. Large lipid droplets stained red, and filled the greater part of the cells (Fig. 1). The staining confirmed that preadipocytes differentiated into mature, functional adipocytes.

Oxygen consumption

The protocol for oxygen consumption rate (OCR) measurement in the intact cells (Fig. 2) included in sequence: (I) a 10-min period of routine respiration (routine), reflecting the aerobic metabolic activity under cellular routine conditions, (II) oligomycin (final concentration 2 µg/ml) -inhibited respiration (oligo), which is caused mainly by compensation for the proton leak after inhibition of ATP synthase; (III) FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydradione) titration (1.5–3.0 µM), which yields the maximum stimulated respiration, as a measure of electron transport system capacity of uncoupled mitochondria; (IV) antimycin A (12 µM) and rotenone (3 µM), that inhibit mitochondrial respiratory complexes III and I, respectively (Fig. 2).

Basal OCR of control and Ex-4 treated cells did not differ significantly. When the non-mitochondrial oxygen consumption, measured after full inhibition of mitochondrial electron transport chain by antimycin A and rotenone, was subtracted, the routine mitochondrial respiration was increased in the Ex-4 treated cells (Fig. 3A). Further analysis after inhibition of the ATP synthase by oligomycin revealed that exendin-4 enhanced non-ATP linked OCR, but did not change ATP-linked OCR (Fig. 3B). After stimulation of the

![Figure 1. Human ChubS7 preadipocytes prior to differentiation (A) and with Oil Red-O staining (C); and ChubS7 cells after 21 days of differentiation into adipocytes without staining (B) and with Oil Red-O staining (D); magnification 200x.](image1)

Intracellular ATP content

The intracellular ATP levels measured by lumimetric method were comparable in the exendin-4 treated cells, exendin-9 treated cells and control adipocytes (Fig. 3D). These results confirm that the observed increased oxygen consumption by mitochondria was not associated with excessive ATP synthesis, although the energy homeostasis was maintained.

![Figure 2. An example of oxygen consumption rate trace (OCR; pmol oxygen per minute per 1x10^6 cells, mean ±S.D. from 5 experiments) of CHUBS7 cells.](image2)
Mitochondrial membrane potential (Δψₘ)

Mitochondrial membrane potential was measured by JC-1 staining. The ratio of red to green fluorescence intensities generated by JC-1 staining was reduced by 17% (n=5, p<0.05) indicating that the mitochondrial membrane potential was slightly decreased in the Ex-4 treated cells when compared to the control. A reduced signal from an orange-fluorescent J-aggregate that is associated with hyperpolarized membrane potentials and indicates mild mitochondrial ETC uncoupling, was recorded in the Ex-4 treated cells (Fig. 4).

Gene expression

Exendin-4 was found to up-regulate two genes coding for sirtuins (class III histone deacetylase): SIRT1 coding for sirtuin 1 and SIRT3 coding for sirtuin 3 (Fig. 5A, B). This effect was abolished when exendin-9 was added to the cells.
Histone deacetylases’ activity

There were no significant differences between the exendin-4 treated and untreated adipocytes in the total activity of histone deacetylases measured in the nuclear extracts (Fig. 5C). Nevertheless, when the trichostatin A, an inhibitor of class I HDAC and class II HDAC, was added to the nuclear samples, the activity of trichostatin A insensitive HDAC was enhanced in the exendin-4 treated adipocytes when compared to the control adipocytes. This indirectly indicates an increased activity of class III histone deacetylases, i.e. the sirtuins (1-7).

DISCUSSION

The effect of GLP-1 receptor signaling in central nervous system on stimulation of brown adipose tissue thermogenesis through adrenergic neurons in rodents is well documented, but these results do not exclude the possibility of direct influence of GLP-1 on adipocyte browning (Lockie et al., 2012; Beiroa et al., 2014). While some reports from the rodent studies confirm this hypothesis (Mostafa et al., 2015; Xu et al., 2016), other reports suggest that increased BAT thermogenesis did not play a significant role in the food intake-independent body weight lowering effect of GLP-1RAs (Heppner et al., 2015; Chac et al., 2015).

A few studies had indicated direct influence of exendin-4 on mitochondrial function. Recently, a GLP-1RA (liraglutide) specific reduction of lipid content in the liver was reported, along with a decreased expression of major elements involved in lipogenesis, peroxisomal β-oxidation and lipid flux (Decara et al., 2016). In the NXS-1 rat pancreatic beta cell line, exendin-4 prevented cytokine induced apoptosis through counter-regulation of the reduced abundance of electron transport chain proteins (Tews et al., 2009). Exendin-4 was also found to increase mitochondrial mass (Kang et al., 2015). A very recent in vitro study had shown that exendin-4 increases basal and fatty acid stimulated oxygen consumption rate, as well as increased expression of thermogenic UCP1 and PPAR-α in differentiated muscle cells (Choung et al., 2017). Exendin-4 improved mitochondrial function by normalization of citrate synthase activity, mitochondrial oxidative phosphorylation and supercomplex formation in mice after heart failure, and the effects were abolished by GLP-1 antagonist, exendin-9 (Takada et al., 2016). Mechanisms by which exendin-4 increases cardiomymoblasts survival also involve improvement of mitochondrial respiration and suppression of the opening of mitochondrial permeability transition pore (DeNicola et al., 2014).

In our study, exendin-4 stimulated the mitochondrial routine and maximal respiration, increased the reserved capacity and preserved intracellular ATP level in the CHUBS7 adipocytes. It is hypothesized that a reserve capacity exists as a compensation mechanism for cells when presented with increased workload or as a stress response (Cannon & Nedergaard, 2004; Dranka et al., 2010). Indeed, uncoupling can be stimulated by either high levels of cellular/mitochondrial ROS or by circulating free fatty acids (FFA) (Jarmuszkiewicz et al., 2004; Sluse et al., 2006). The effects of exendin-4 on mitochondrial membrane potential and mild uncoupling reported here, may explain the protective role of GLP-1 (secreted physiologically in response to food intake) to adapt to the metabolic stress occurring in the postprandial period.

In the study presented here, exendin-4 up-regulated mRNA expression of SIRT1 and SIRT3, along with the bioenergetic effects in the CHUBS7 human adipocyte cell line. Sirtuins belong to class III HDAC and thus regulate a number of key metabolic enzymes that are acetylated, malonlated, or succinylated in mitochondria in response to environmental stimuli (Imai et al., 2010; Chalkedaki et al., 2012). Sirtuin 1 (Sirt1) extends lifespan and is involved in energy metabolism (Frye, 2000). Sirtuin 3 (Sirt3), preferentially localized in mitochondria, targets enzymes of the respiratory chain, tricarboxylic acid cycle, fatty acid β-oxidation and ketogenesis (Hirschey et al., 2010; Giralt & Villarroya, 2012). Our results for SIRT1 and SIRT3 expression are in line with a recent report that exendin-4 up-regulates adiponectin in adipocytes through the Sirt1/Foxo-1 signaling pathway (Wang et al., 2017). Similarly, exendin-4 increased lipolysis and fatty acid oxidation by up-regulating SIRT1 expression and activity in differentiated 3T3-L1 adipocytes (Xu et al., 2016). These findings, along with our results, give the basis for the hypothesis that GLP-1 agonist may promote brown remodeling of WAT in a SIRT1-dependent manner. Discovery of the involvement of sirtuin 1 in the exendin-4 mechanism of action in adipocytes is of great importance in the light of data confirming depleted sirtuin levels in obesity (Mariani et al., 2016).

In our study, exendin-4 not only up-regulated SIRT1 and SIRT3 mRNA expression in the CHUBS7 adipocytes, but also increased the class III HDAC activity, although total HDAC activity was not affected. In a recent paper, total HDAC activity was reported to be decreased in visceral adipose tissue of obese human subjects when compared to normal weight subjects. Decreased Hdc5 and Hdc6 expression has been shown in adipocytes from obese human subjects, as well as from obese mice (Bricambert et al., 2016). Activation of sirtuins by exendin-4 was also observed in hepatocytes and rat retinal cells (Lee et al., 2014; Zeng et al., 2016). Thus the restoration of proper sirtuin activity may be one of the mechanisms of pleiotropic effects of GLP-1RAs.

Recently, there has been observed a growing interest in inducible brown-like adipocytes (“beige”) in white adipose tissue depots in response to appropriate stimuli. Beige adipocytes have characteristics similar to brown adipocytes, in particular the capacity for uncoupled respiration. Utilization of nutrients by beige adipocytes improves insulin sensitivity and glucose tolerance, but their number in overweight and obese individuals is reduced (Loyd & Obici, 2014). Thus, induction of browning of the white fat represents an attractive potential strategy for the management and treatment of obesity and related complications (Bonet et al., 2013). In this paper, we have found that exendin-4 stimulates oxygen consumption and mitochondrial uncoupling in human adipocytes, and thus switches mitochondrial bioenergetics towards energy expenditure. Detailed studies on human adipose tissue are needed to confirm the initial hypothesis that exendin-4 could directly induce browning of the white adipose tissue in humans.

This paper makes contribution to the field of study on the pleiotropic effects of incretin mimetics. The results indicate stimulation of mitochondrial bioenergetics as the effect of exendin-4 action in human adipocytes. Enhanced expression of sirtuins (1 and 3) may elucidate the mechanism of exendin-4 action in human adipocytes, although further studies are needed to investigate the causal relationship of sirtuin and mitochondrial respiration in this model. The results contribute to the understanding of the GLP-1RAs induced weight loss in patients, which results not only from a decreased energy intake but also from an increased resting energy expenditure.
Conflicts of interest

The authors declare that they have no conflicts of interest.

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