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Research Article

GLP-1 Effects on Adiponectin and IL-6 Expression in 3T3-L1 Adipocytes: Obesity and Inflammation Study

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Abstract

Diabetes Mellitus Type 2 (T2DM) and obesity have rapidly increased in incidence, becoming two of the most devastating health issues worldwide. The understanding of T2DM, obesity and inflammatory-related diseases has developed significantly over the past two decades, thus providing new insights and understanding of the pathophysiology and mechanisms involved, with adipose tissue inflammation and insulin resistance being a common denominator in each. Adipose tissue is a complex organ which, due to its role in the secretion of pro and anti-inflammatory adipokines, such as IL-6 and adiponectin, is now considered as an endocrine system, with these adipokines being involved in the low-grade chronic inflammation observed in response to increased adiposity and impaired insulin signalling. Glucagon-like-peptide-1 is an incretin which is regarded for its insulinotropic effect. Due to this GLP-1 and its analogues are emerging as effective treatments for T2DM. However, its role in reducing inflammation in AT has yet to be widely investigated. This in vitro 3T3-L1 adipocyte cell considers the actions of GLP-1 on the secretion of IL-6 and adiponectin. Results from the study indicated a significant effect in down-regulating the expression of proinflammatory adipokine IL-6 in 3T3-L1 adipocytes, with no significant changes in the expression of anti-inflammatory adipokine adiponectin. In conclusion, this study indicates that GLP-1 can reduce the expression of IL-6 in 3T3-L1 adipocytes; the mechanisms and pathways involved require further investigation. Further understanding of the interplay of GLP-1, AT and adipokines could lead to improvements in the treatment of T2DM, obesity-related inflammation and-related disease with improved insulin sensitivity.

Keywords: Diabetes; Obesity; Adipokines; Adipose tissue

Introduction

There are 422 million people living with diabetes globally, with 90% of them having Type 2 Diabetes (T2DM), which has increased fourfold since 1980. While lifestyle and pharmacological interventions can prevent T2DM, genetic factors such as ethnicity may also contribute to its development, particularly in South Asian, African, and African Caribbean populations [1]. Diabetes caused 1.6 million deaths in 2015 and is projected to affect 4 million people in the UK by 2025, with childhood incidence also increasing [2]. CVD is the leading diabetes-related illness, responsible for 80% of deaths. In the UK, T2DM cost the NHS GBP 11.718 billion in 2012 due to diabetes-related illnesses [3]. T2DM risk factors consist of obesity, sedentary lifestyles, increased blood pressure, smoking, and elevated fat and cholesterol levels. T2DM is a chronic metabolic disease that progresses gradually, and its symptoms may not be immediately apparent, causing difficulty in diagnosis. This often leads to a more advanced stage of the disease upon diagnosis. Early interventions have a positive impact on outcomes and lower the prevalence of diabetes-related illnesses, according to Herman, et al. (2015) [4]. The abnormal or excessive accumulation of adipose tissue, known as obesity, is a major global health concern with serious implications for health, as defined by the World Health Organisation [5]. Obesity and its associated health conditions are considered the epidemic of the 21st century due to a significant increase in its prevalence over the past 30 years. Currently, it is estimated that 650 million adults worldwide are obese, and

1.9 billion are overweight. The most common methods used to diagnose obesity are the Body Mass Index (BMI) [6] and waist-to-hip ratio [7]. In the UK, the prevalence of obesity has risen by 12% between 1993 and 2015, with 27% of adults classified as obese (BMI of 30^{+}), and an additional 36% overweight (BMI 25–29.9) [8]. Projections indicate that more than half of the population will be obese by 2050 [9]. In 2015/2016, obesity-related healthcare costs to the NHS were estimated at GBP 6.1 billion, with estimates predicting this figure to rise to GBP 9.1 billion by 2050, according to Public Health England. In many cases, external factors such as diet and physical activity can contribute to the development of obesity through an energy imbalance caused by consuming more calories than are burned. This results in excess energy being stored as triglycerides in adipocytes to decrease the levels of circulating free fatty acids in the bloodstream. High levels of stored energy can lead to adiposity and dysfunctional adipocytes, which can affect the regulation of Adipose Tissue (AT). Researchers are investigating specific genes and hormones to gain a better understanding of the interactions between AT, the central nervous system, and the endocrine system, and how they contribute to the onset of chronic low-grade inflammation. For example, Chabot, et al. (2014) [10] explored the relationship between ghrelin and insulin resistance and how this may impact metabolic functions and inflammation. There is a scarcity of research investigating the direct impact of GLP-1 or incretins on adipokine expression in Adipose Tissue (AT) or 3T3-L1 adipocytes. While research on the role of GLP-1 and its receptors has primarily focused on hepatocytes, the central nervous system, and skeletal muscle, AT has not been extensively studied [11,12]. Nonetheless, the literature suggests that GLP-1 receptor agonists have the potential to reduce inflammation, insulin resistance, and diabetes by altering adipokine expression, as demonstrated by in vitro and in vivo studies. Vendrell, et al. (2011) [11] suggested that there may be a connection between insulin resistance, obesity, and GLP-1 receptors in AT. It was also observed that treatment of RAW cells with GLP-1 did not cause a significant reduction in adiponectin secretion by 3T3-L1 adipocytes when considered alone. However, a decrease in expression was noted when compared to co-cultured cells without GLP-1 treatment. According to Vendrell, et al. (2011) [11] indicates that GLP-1 can counteract the inhibitory impact of macrophage infiltration on adiponectin. It is debatable whether this result can be regarded as conclusive evidence, as other studies, including Shirashi, et al. 2012 [13], have shown no significant influence of GLP-1 treatment on adiponectin expression. Nonetheless, it suggests that GLP-1 may offer a protective effect in the presence of macrophages. Current research suggests that there is a correlation between adipokine secretion and immune cell response that affects the inflammatory response involved in regulating insulin sensitivity. GLP-1 appears to mediate this interaction in the presence of obesity. However, there is a lack of research on the role of GLP-1 in AT, even in the presence of GLP-1R, and its potential to improve understanding of the pathophysiology of inflammation and insulin resistance. As discussed earlier, the devastating effects of obesity and T2DM on global health are evident, and their connection to inflammation, insulin resistance, obesity, and the role of adiponectin and IL-6 is well-established. Despite this, the impact of GLP-1 on adipocytes and adipokine expression, which play an active role in the inflammatory response and obesity-related diseases, has not been thoroughly studied. To address this gap in knowledge, the present study was conducted to investigate the anti- or pro-inflammatory effect of GLP-1 on the protein expression of adipokines, specifically adiponectin and IL-6, in 3T3-L1 differentiated adipocytes using ELISA immunoassay at both 4 and 24 h.

Materials and methods

Experimental design and materials

3T3-L1 embryonic mouse cells were cultured and then differentiated to adipocytes; once the differentiated adipocytes cells reached confluence, they were treated with GLP-1, as described in detail below. Following treatment ELISA immunoassay was used, respectively, to read the adiponectin and IL-6 absorbance levels of the differentiated cell supernatant.

T3-L1 Cell culture

3T3-L1 fibroblasts (Sigma-Aldrich; Dorset, UK) were grown in culture medium made up of Dulbecco's Modified Eagle's Serum (DMEM) (Sigma-Aldrich; Dorset, UK) containing 10% Foetal Bovine Serum (FBS) (Gibco, ThermoFisher Scientific; Rochford, UK), 1% l-glutamine (Gibco, ThermoFisher Scientific; Rochford., UK) and 1% PenStrep (Gibco, ThermoFisher Scientific; Rochford, UK). The preadipocyte cells were incubated at 37.5°C, 5% CO_2 and routinely maintained every 48-72 h, with media being aspirated and replaced with 2ml of fresh culture medium. Proliferation observed until the fibroblasts reached 70–80% confluence (70–80% of the base of flask covered with adhered cells).

At 70–80% confluence the cells were 'split' to encourage increased cell growth. To split the cells the medium was removed from the flasks and 1ml of Trypsin added (Sigma-Aldrich; Dorset, UK). When cells reached confluence, differentiation was induced. Induction day was described as day 0. Cells were counted using a haemocytometer; 100µL of 0.4% trypan blue stain (Sigma-Aldrich; Dorset, UK) was mixed with an equal amount of cell suspension;cell suspension was derived as previously mentioned, and cells counted (3 squares counted on haemocyte grid to give an accurate cell count). Cells were then plated in to 6well plates, with 2 × 104 cells in each well; 8 plates were used in total.

Differentiation was induced by using differentiating medium containing DMEM with 10% FCS with the addition of 0.25 µM of dexamethasone (Calbiochem; Notts, UK), 0.5 Mm of Isobutyl Methylxanthine (IBMX) (Sigma-Aldrich; Dorset, UK), and 5 µg/mL and 10mg/mL of insulin (BD Bioscience); 2 mL was added to each well [14].

Treatment of fully differentiated 3T3-L1 adipocytes with GLP-1

Treatment took place on day 14 post-adipocyte differentiation by adding 100 nM of GLP-1 (Peptides International) to cell culture medium with the addition of 5 µg/mL of insulin, as previously described. The medium containing GLP-1 was added to 24 wells; the medium without GLP-1 was added to another 24 wells for controls. Cells were then incubated at 37.5°C and 5% CO_2 until being harvested at 4 and 24 h, respectively. Cell media were aspirated from wells and centrifuged at 200 × g for 10 min at room temperature (Sigma-Aldrich; Dorset, UK) [15].

Immunoassay Using ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was used for the detection of both Adiponectin (Invitrogen; CA, USA), and IL-6 (ELISA MAX Deluxe Sets; Biolegend, Inc., San Diego, CA, USA). Absorbance readings were taken at 450 nm within 30 min of stop solution being added to plates. A BMG Labtech fluostar Omega plate reader was used, and the results were adjusted for blanks.

Statistical analysis

SPSS was used to complete an unpaired T-test comparing mean values of control groups to that of treatment groups for both adipokines separately displayed adiponectin and IL-6. p values of <0.05 were considered to show statistical significance.

Results

GLP-1 Downregulates IL-6 Expression in 3T3-L1 Adipocytes

IL-6 control samples gave a standard curve equation of y = 0.0016x + 0.3573 with R2 = 0.9882. The results show that after 4 h treatment with GLP-1 there was a statistical significance in the down regulation of expression of IL-6 compared to control group p = 0.003 (Figure 1). With S.D. of 4 h control ± 92.9 and 4 h treatment ± 18.62, mean values, respectively.

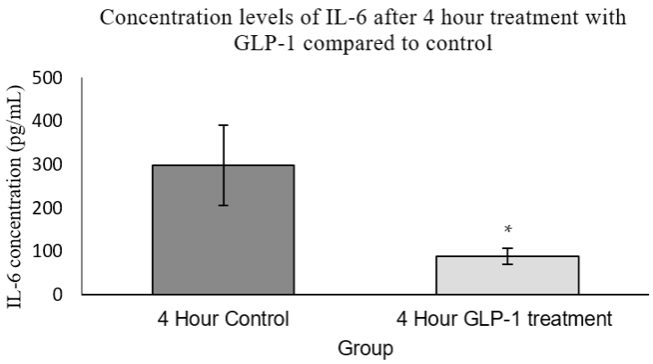

Figure 1: Mean concentrations (pg/mL) of IL-6 expression from 3T3-L1 adipocytes after 4 h treatment with GLP-1 compared with control group. O.D. (450nm) was analysed using ELISA immunoassay. Data shown represent mean values ±. S.D. *Statistically Significant $p \leq 0.05$ compared to control. p = 0.001.

After 24 h of treatment with GLP-1, compared to control, there was also a statistical significance p = 0.01 in the downregulation of IL-6 expression (Figure 2).

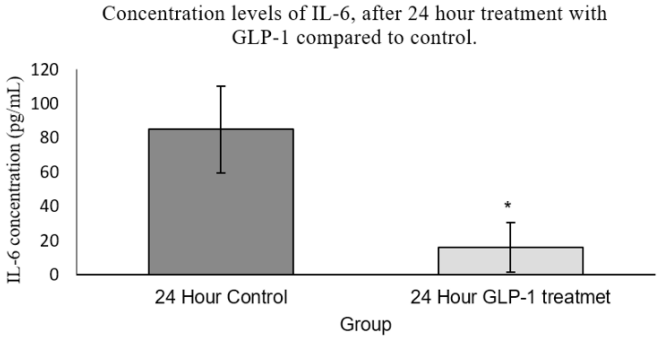

Figure 2: Mean concentrations (pg/mL) of IL-6 expression from 3T3-L1 adipocytes after 24 h of treatment with GLP-1 compared with control group. O.D. (450 nm) was analysed using ELISA immunoassay. Data shown represent mean values ±. S.D. *Statistically significant: $p \leq 0.05$ compared to control.

To further analyse the significant effect of GLP-1 treatment on the expression of IL-6 in 3T3-L1 adipocytes, the mean concentration (pg/mL) of 4 h treatment samples was compared with the 24 h treatment sample, the result shows that treatment over a longer period had a greater impact on the down regulation of IL-6 in 3T3-L1 adipocytes (Figure 3): S.D. 24 h control ± 25.31 *vs.* 24 h treatment ± 14.71.

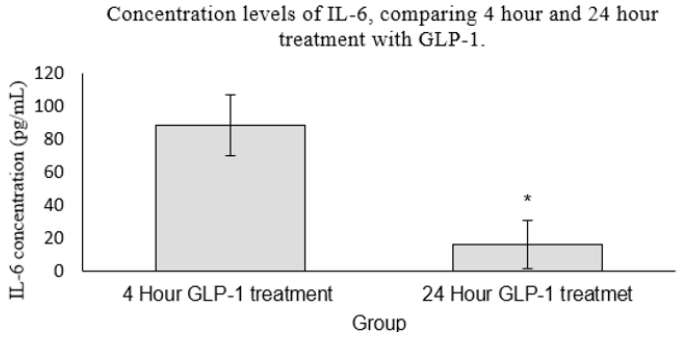

Figure 3: Mean concentrations (pg/mL) of IL-6 expression from 3T3-L1 adipocytes comparing 4 and 24 hours of treatment with GLP-1. O.D. (450 nm) was analysed using ELISA immunoassay. Data shown represent mean values ±. S.D. *Statistically significant $p \leq 0.05$ compared to control.

GLP-1 has no Effect on Adiponectin Secretion in 3T3-L1 Adipocytes

Adiponectin control samples gave a standard curve equation of y = 0.2111x+0.5143 with R2 = 0.9004. Adiponectin expression from adipocytes displays anti-inflammatory properties; if these could be enhanced using GLP-1, this may lead to an overall reduction in inflammation, attenuating improved insulin resistance, especially in T2DM. However, the results show that following both 4 and 24 h of treatment with GLP-1, there was no significant effect on adiponectin expression in 3T3-L1 adipocytes when compared with control groups ($p \geq 0.05$) (Figures 4 and 5). Concentration levels of adiponectin were slightly increased after the treatment of GLP-1; however, the effect was minimal.

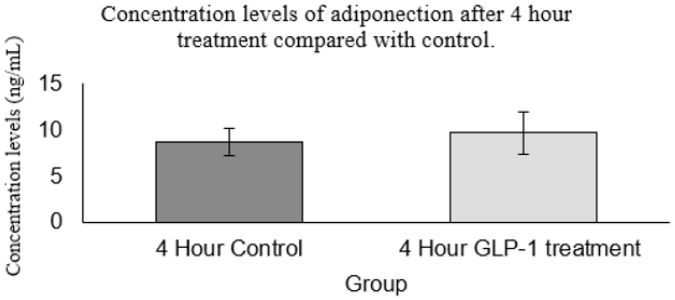

Figure 4: Mean concentrations (ng/mL) of adiponectin expression from 3T3-L1 adipocytes after 4 h of treatment with GLP-1 compared with control group. O.D. (450nm) was analysed using ELISA immunoassay. Data shown represent mean values ±. S.D. p = 0.443.

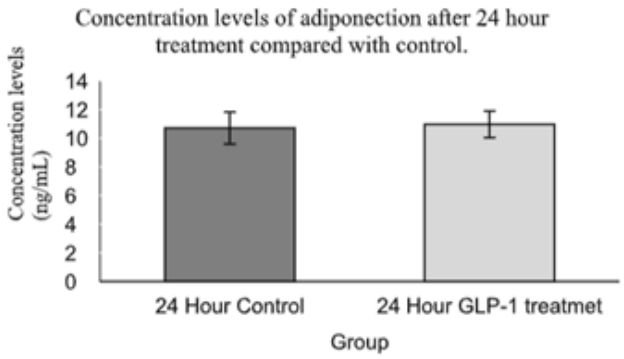

Figure 5: Mean concentrations (ng/mL) of adiponectin expression from 3T3-L1 adipocytes after 24 h of treatment with GLP-1 compared with control group. O.D. (450 nm) was analyzed using ELISA immunoassay. Data shown represent mean values ±. S.D. p = 0.765.

A trend could be seen in a slight increase in adiponectin expression in the presence of GLP-1 treatment in 3T3-L1 adipocytes over a longer amount of time; the mean concentration (pg/mL) of the 4 h treatment samples was compared with the 24 h treatment sample (Figure 6) (mean values: 9.67 ng/mL and 10.97 ng/mL, respectively). However, no significance was shown between expression when comparing the 4 h treatment to the 24 h treatment. The 24 h treatment displayed a smaller S.D. of ± 0.93, compared with the ± 2.28 of the 4 h treatment.

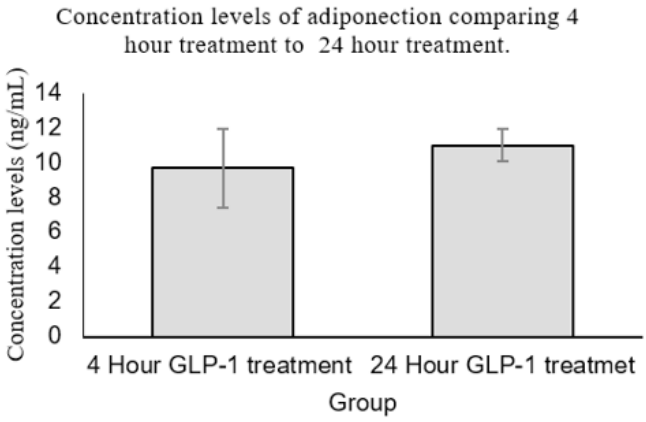

Figure 6: Mean concentrations (ng/mL) of adiponectin expression from 3T3-L1 adipocytes, comparing 4 h and 24 h of treatment with GLP-1. O.D. (450 nm) was analysed using ELISA immunoassay. Data shown represent mean values ±. S.D. p = 0.281.

Discussion

In the present novel study, it was demonstrated that the treatment of 100nM of GLP-1 downregulates the expression of IL-6 in 3T3-L1 adipocytes. GLP-1 significantly reduced the protein expression of IL-6 when compared with untreated controls after both 4 and 24 h treatments. This effect agrees with previous studies which report down regulation of IL-6 expression in the presence of GLP-1 receptor agonists [16]. IL-6 produced in the AT is classified as pro-inflammatory, so the reduced expression observed in this study indicates that GLP-1 reduces the inflammatory response in AT.

On the other hand, it is known that adiponectin is an anti-inflammatory cytokine, the expression of which is decreased in the presence of obesity. An increase in the expression of adiponectin would indicate an anti-inflammatory response negating the effects of inflammation in AT. This study showed that GLP-1 treatment had no significant effect on the expression of adiponectin. A trend was observed in a time-dependent manner; adiponectin expression displayed a slight increase in concentration (ng/mL) following both 4 and 24 h of treatment when compared with un-treated controls, and then, with a comparison of both treatment groups, this increase was not statistically significant. This indicates that GLP-1 does not increase an anti-inflammatory response in 3T3-L1 adipocytes. This again agrees with previous studies considering this response [13,17]. In contrast to this, Chung, et al. 2009 indicated that the secretion of adiponectin mRNA was increased in the presence of Exedin-4 through increasing the GLP-1 response suggesting that Exedin-4 may reduce inflammation in adipocytes [16]. It can be taken from the results that adiponectin neither reduced nor increased in the presence of GLP-1.

The GLP-1 mechanism within adipocytes is not yet known Vendrell, et al. 2011 highlighted the presence of GLP-1 receptors in AT, and it can be understood that GLP-1 will respond in adipocytes in AT similarly to in the pancreas, through the binding to GLP-1 to GLP-1R, in the pancreas binding increases the activation of cAMP production. This is demonstrated using GLP-1 agonists [11]. Exedin-4 GLP-1 agonist was used in the study by Wang, et al. 2017 which showed significant cAMP induction following treatment, it was suggested that the increased cAMP levels blocked the expression of adiponectin in response to Exedin-4 in 3T3-L1 adipocytes; this hypothesis may explain the lack of significance in the increase in expression of adiponectin following GLP-1 treatment [18]. Exedin-4 has a longer half-life than GLP-1 of 18-41 min [19]. This may explain the differing results between the present study and the Chung et al. 2010 study in terms of adiponectin expression, whereby the study claimed that there is the possibility of degrading GLP-1 by DPP-IV, which affected the response of adiponectin.

The results of the present study suggest that the observed effect on IL-6 expression is unlikely to be explained by DPP-IV inhibiting action alone. One possible explanation is that GLP-1 may affect IL-6 expression prior to the inhibitory action of DPP-IV, while the expression of adiponectin may require a longer period of exposure to GLP-1 to elicit a response. This could be investigated by monitoring the response of GLP-1 on adipokine expression after much shorter treatment times such as 0–5 min; this would highlight any acute response to GLP-1; this should also be compared to longer treatment times, as a reduction in overall chronic low-grade inflammation is needed to reduce obesity-related inflammatory disease, not just an acute response.

In a study conducted by Dobrain, et al. in 2010, the effect of DPP-IV inhibitors was investigated in both adipose tissue and pancreatic islets. The researchers found that sitagliptin, a DPP-IV inhibitor, caused a similar reduction in IL-6 expression in pancreatic islets as observed in adipocytes in the present study [20]. Their *in vivo* study also showed a significant decrease in IL-6 mRNA expression in mice fed a high-fat diet and treated with sitagliptin [20].

To make more accurate comparisons, it is suggested to differentiate lean and obese cells based on their level of adiposity. This can be achieved by loading differentiated 3T3-L1 adipocytes with lipids and using real-time PCR to determine the amount of Adipogenesis-Promoting Nuclear Factor (PPARγ) present. This approach can also be extended to induce hypertrophy in cells and investigate the effect of GLP-1 on the expression of adipokines in the presence of different levels of adiposity [21]. These studies can then be extended to *in vivo* experiments by comparing lean and obese mice, and ultimately, human subjects, as previously performed by Lee, et al. (2012) and Bekay et al. (2016), respectively [22,23].

It is important to note that adiponectin and IL-6 are only two of the many adipokines expressed in AT. Since TNF-α was under-expressed when replicated in vivo, and hyperleptinemia, leptin resistance, and unresponsiveness to leptin therapy are observed in obesity [24-26] they were not considered in this study. The study focused only on adiponectin and IL-6 as inflammatory adipokines. However, it is worth mentioning that the study by Rotter, et al. (2003) showed that IL-6 and TNF-α have different effects on the JNK inflammatory-initiated pathway, which is involved in insulin resistance, and that TNF-α increases IL-6 secretion [17].

Although the exact mechanisms of GLP-1 in AT inflammation along with insulin resistance are not yet fully known, this study demonstrates positive evidence that GLP-1 treatment can be successfully used to reduce the expression of proinflammatory cytokines (IL-6) in 3T3-L1 adipocytes, which as described play a role in initiation of the pathways involved in the development and progression, of T2DM, inflammation, insulin resistance, and the onset of obesity-related disorders. Knowledge from this, alongside previous relevant studies, supports the notion that GLP-1 inhibits the inflammatory response through a reduction in inflammatory adipokines but does not display an effect on anti-inflammatory

adipokines. Further studies into the mechanisms by which GLP-1 attenuates this response, as described, could lead to further understanding of how GLP-1 can be utilized as a therapeutic agent, opening the doors to establishing successful treatments to reduced obesity induce insulin resistance, inflammation, and T2DM.

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Conflicts of Interest: The authors declare no conflict of interest.

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