THE EFFECT OF SALBUTAMOL ON PGC-1 α AND GLUT4 mRNA EXPRESSION IN THE LIVER AND MUSCLE OF ELDERLY DIABETIC MICE

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Abstract

Background. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) plays an important role in the regulation of cellular energy metabolism, and it is involved in obesity and type 2 diabetes mellitus (T2DM). Its expression is elevated in the liver of T2DM mouse models. Literature reports show that chronic β 2 stimulation improved insulin sensitivity in T2DM.

Objectives. We aimed to test the hypotheses that chronic β 2 stimulation-induced improvement in insulin sensitivity involves changes in the expression of PGC-1 α and glucose transporter 4 (GLUT4).

Animals and Methods. We fed a locally inbred, 8 months old mice, a high fat diet (HFD) to induce diabetes. These mice gained weight and became insulin resistant. The β 2 agonist salbutamol had a beneficial effect on both glucose tolerance and insulin sensitivity after 4 weeks.

Results. Salbutamol beneficial effect persisted after 4 weeks of its discontinuation. HFD caused an up regulation of the hepatic PGC-1 α expression by 5.23 folds ($P < 0.041$) and salbutamol reversed this effect and caused a down regulation by 30.3 folds ($P < 0.0001$). PGC-1 α and GLUT4 expression in the muscle was not affected by salbutamol ($P > 0.05$).

Conclusion. Down regulation of the liver's PGC-1 α contributes to the beneficial effect of the chronic β 2 stimulation on glucose tolerance and insulin sensitivity in T2DM mice.

Key words: diabetic mice, salbutamol, PGC-1 α .

INTRODUCTION

Type 2 diabetes mellitus (DM) is a metabolic disorder of glucose homeostasis associated with long-term vascular complications leading to morbidity and mortality. The combination of insulin resistance and β cell defects in glucose-stimulated insulin secretion causes the impaired glucose tolerance to occur (1). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) is a transcriptional

coactivator that regulates the genes involved in energy metabolism (2). It promotes the transcription of nuclear genes that encode mitochondrial fatty-acid oxidation enzymes and regulate muscle fiber type determination (3). Moreover, PGC-1 α increases GLUT4 mRNA expression and mitochondrial oxidative metabolism (4). GLUT4 is the predominant isoform of the glucose transporters in skeletal muscles (5). Insulin regulates GLUT4 translocation from intracellular pool to cell surface, where it facilitates the entry of glucose inside the cell (6). However, most studies have failed to demonstrate reduced GLUT4 expression levels in skeletal muscle of type 2 diabetic patients (7, 8).

NAD⁺-stimulated Sirtuin 1 (SIRT1) mediated deacetylation is required for PGC-1 α coactivation of gluconeogenesis and fatty acid oxidation in the liver (9, 10). SIRT1 is up-regulated after fasting or calorie restriction (11). The activation of hepatic SIRT1 leads to PGC-1 α deacetylation, which increases gluconeogenic genes and represses glycolysis (9). After refeeding, the nutrient-induced rise in insulin leads to increased expression of general control of amino acid synthesis 5 (GCN5), which acetylates and thereby inactivates PGC-1 α , leading to repression in gluconeogenesis, fatty acid oxidation and oxidative phosphorylation (12). Koo and coworkers demonstrated that PGC-1 α deficient mice experience fasting hypoglycemia and enhanced liver insulin sensitivity (13). Therefore, it has been suggested that PGC-1 α affects insulin sensitivity negatively in the liver (14).

Epinephrine (EP) is a stress hormone (15). EP facilitates insulin binding to exercising muscle (16) and increases muscle blood flow. Although, the β -adrenergic agonist EP acutely increases glucose and decreases insulin sensitivity, chronic E infusion improves insulin sensitivity (16). Injecting a drug that mimics the effects of E in mice increases the transcription of PGC-1 α in the muscle through the second messenger cyclic adenosine monophosphate

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(cAMP), which increases mitochondrial biogenesis and promotes the formation of new blood vessels in muscle (17). However, the mechanisms by which E and β_2 -agonists might stimulate skeletal muscle insulin sensitivity are not well understood, but increased muscle energy utilization and increased translocation of the glucose transporter GLUT4 could explain certain routes (18).

Elayan and coworkers reported that the administration of the β_2 adrenergic agonist, salbutamol in old diabetic mice, improved glucose tolerance, while exercise did not. They conducted their experiment using eight-month old mice. This age matches the middle age in man, which is the age that type 2 DM develops. They suggested that the lack of response to exercise is consistent with a lack of endogenous β_2 stimulation in older diabetic animals, but the response to a β_2 -agonist in older diabetic animals suggests that this can be overcome pharmacologically (19). EP release is diminished in the elderly (20) especially during exercise (21).

Since the role of GLUT4 in the pathogenesis of type 2 diabetes has not been clearly established yet (22 & 23) and PGC-1 α is a transcriptional coactivator that regulates the genes involved in energy metabolism (2), we aimed at investigating the roles of these two factors in β_2 -adrenergic receptor stimulation induced improvement in insulin sensitivity in 8 month old high fat diet induced type 2 diabetes mellitus.

METHODS

Animals

Thirty six locally inbred 8 month old male mice, with an average weight of 28.3 ± 0.52 g, were used in this experiment. All experimental work was carried out according to the guidelines of UJ ethical committee (approval no.: 5/3/1/2687, date: 18/05/2013).

Diet preparation

Several diet mixtures containing 60% fat were prepared. The diet mixture that achieved a weight gain in a pilot study was the one used throughout the whole experiment. It contained 60% animal fat (sheep) mixed with 40% crushed feed (Hammoudeh Food Industries Co., Amman, Jordan) (19).

Protocol

The experiment started with thirty six mice; twenty four mice were fed with a high fat diet (HFD, 60% fat) until the end of the experiment and twelve mice were fed with a normal mouse diet and served as

controls. Ten of the controls remained till the end of the experiment. All mice were weighed at the end of every month.

Toward the end of 13 weeks of high fat feeding, 6 mice out of 24 died. The remaining 18 mice were divided into two groups, 9 mice received 5% of the selective β_2 -adrenoceptor agonist salbutamol (Asmadil 2, the Arab Pharmaceutical Manufacturing Co., Ltd., Jordan) dissolved in their drinking water for 4 weeks (HFD+Salbutamol group), and the other 9 mice (HFD group) continued to drink tap water (19). Both groups continued the high fat feeding till the end of the experiment.

Assessment of insulin resistance and glucose intolerance

Intraperitoneal (i.p.) glucose tolerance tests (GTT) and i.p. insulin tolerance tests (ITT) were performed on mice after 6 hours of fasting. One drop of tail blood from each mouse was used to estimate the blood glucose concentration per unit time using a glucose meter (Prodigy, Prodigy diabetes care Inc., UK). The GTT was performed by i.p. injection of 25 % dextrose (25% w/v dextrose, Pharmaceutical Solution Industries) with a dose of 2g / kg body weight in a total volume of 0.2 mL, and the blood glucose concentration was measured at 0, 15, 30, 60, 90 and 120 minutes from the time of the injection (19).

The ITT was performed by i.p. injection of insulin (100 IU/mL Insulin vial, Actrapid 10 mL, Novonordisk) in a dose of 0.85U/Kg body weight in a total volume of 0.15 mL. The blood glucose concentration was measured at 0, 15, 30, 60, 90 and 120 minutes after the injection (19).

Muscle and liver biopsies

After four weeks of Salbutamol treatments, a biopsy was taken from the right leg of each mouse of all the mice in the three groups (Control, HFD, HFD+Salbutamol). Each mouse's right leg was opened using scissors under ether anesthesia and the biopsy was taken from the soleus muscle using biceps and a cutter. The muscle was weighed and stored at -80°C.

Four weeks later, all the mice were sacrificed by decapitation and a second muscle biopsy was taken from the left leg of each mouse using the same method and storage as mentioned before. At the time of the decapitation the liver was also collected from each mouse, washed with a 0.9% sodium chloride solution to remove the blood contained in the tissue, and stored at -80°C.

RNA extraction and cDNA synthesis

Total RNA was extracted from the soles muscle using Trizol Reagent (One step RNA Trizol reagent, Bio Basic INC.), according to the manufacturer's instructions. The first strand of cDNA was synthesized from 1 µg of total RNA using the reverse aid first strand cDNA synthesis kit (Thermo, EU.).

Quantitative polymerase chain reaction (qPCR)

The gene expression was analyzed using real time PCR (IQ4 Biorad), with 2µl of cDNA for PGC-1α and Beta actin primers. SYBr Green master mix (GoTagqPCR master mix, Promega, USA) was used in a final reaction volume of 25 µL. All samples were run in duplicates with a standard curve in each run. The reaction started with an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for PGC-1 α and at 61°C for Beta actin for 20 seconds and extension at 72°C for 20 seconds. The mRNA levels of PGC-1α were normalized to the mRNA levels of Beta actin. The PGC-1 α and GLUT4 primers were designed according to Nagatomo *et al.*, 2011(23), and the beta actin primer was designed according to ShineGene Molecular Biotech, Inc.. The following primer sequences were used: PGC-1α forward, 5'-CGATGACCTCTCTCACA CCA-3' PGC-1α reverse, 5'-TTGGCTTGAGCA TGTTGCG-3' GLUT4 forward, 5'-CAACTGGACC TGTAACCTTCATCGT-3' GLUT4 reverse, 5'-ACGGC AAATAGAAGGAAGACGTA-3' Beta actin forward, 5'-GTCCCTCACCCTCCCAAAAG- 3' Beta actin reverse, 5'-GCTGCCTCAACACCTCAACCC-3'.

Statistical analysis

All values were presented as mean ± standard error of the mean (SEM). The area under the curve (AUC) for the glucose concentration *versus* time during the ITT and GTT was calculated using the trapezoidal rule and one-way analysis of variance (ANOVA) was used to determine the significance between the mice groups.

The $\Delta\Delta C_t$ method (delta delta cycle threshold) was used to calculate the folds by which the genes were up-regulated or down-regulated in quantitative polymerase chain reaction (qPCR).

ΔC_t control = C_t (target gene control) – C_t (housekeeping gene control)

ΔC_t treated = C_t (target gene treated group) – C_t (housekeeping gene treated group)

$\Delta\Delta C_t = \Delta C_t$ (control) – ΔC_t (treated)

RESULTS

Blood glucose results

After 13 weeks of high fat feeding, the HFD group showed a 22.6% increase in weight ($P < 0.0001$), while the control group's weight increased by 8.8%. The mean weight of the HFD group was significantly higher than that of the control group, ($P < 0.036$).

The GTT and ITT tests were performed every 4 weeks, in all groups, to assess the development of glucose tolerance and insulin resistance. After 13 weeks of HFD feeding, the HFD group showed a significant increase in blood glucose level in GTT and ITT tests ($P < 0.001$ and ($P < 0.0001$, respectively, Fig. 1). Moreover, the ITT revealed insulin resistance that was clear 15 minutes after insulin injection; as the blood glucose increased instead of the expected decrease.

Figure 1. ITT and GTT results showing the mean ± SEM blood glucose concentrations (mg/dL) of the HFD (n=18) and the control (n=10) groups after 13 weeks of high fat feeding.

After insulin resistance and glucose intolerance were developed in the HFD group, nine mice of that group received salbutamol in their drinking water for 4 weeks and a significant decrease in blood glucose concentration in GTT was observed ($P < 0.0001$, Fig. 2). The HFD+Salbutamol group did not show the initial increase in blood glucose in response to insulin injection induced pain during the ITT ($P < 0.0001$, Fig. 2) as the HFD group did.

Although salbutamol administration was stopped for 4 weeks, its beneficial effects on glucose profile persisted as the GTT revealed significant differences in blood glucose levels between the HFD+Salbutamol and HFD groups ($P < 0.023$). Similarly, ITT showed a significant difference in the blood glucose level between the two groups ($P < 0.016$, Fig. 3).

Quantitative polymerase chain reaction (qPCR) results

After 4 weeks of salbutamol administration, PGC-1 α expression in the muscle of the HFD+Salbutamol and the HFD group was not significantly affected. However, after 4 weeks of salbutamol discontinuation, the muscle PGC-1 α in both groups decreased compared to the control (Fig. 4). The HFD showed a decrease in muscle PGC-1 α of 5.54 folds ($P < 0.0001$), while the HFD+Salbutamol group showed a 5.29 decrease in muscle PGC-1 α ($P < 0.0001$).

The hepatic PGC-1 α expression of the HFD group was increased by 5.23 folds compared to control ($P < 0.041$) while the PGC-1 α expression in the liver of the HFD+Salbutamol group was decreased by 30.3 folds compared to control ($P < 0.0001$, Fig.5).

Figure 2. GTT and ITT results showing the mean $\pm$ SEM blood glucose concentrations (mg/dL) of the HFD (n=7) and the HFD + Salbutamol (n=9) group, after 4 weeks of salbutamol administration.

Figure 3. GTT and ITT results showing the mean $\pm$ SEM blood glucose concentrations (mg/dL) of the HFD (n=6) and the HFD + Salbutamol (n=9) group, after 4 weeks of salbutamol administration.

Figure 4. PGC-1 α expression in the soleus muscle of the HFD (n=6) and the HFD+Salbutamol (n=9) group after 4 weeks of salbutamol administration and 4 weeks after Salbutamol discontinuation. * HFD+S: HFD+Salbutamol.

Figure 5. The expression of PGC-1 α in the liver of the HFD (n=6) and the HFD+Salbutamol (n=8) groups after 21 weeks of high fat feeding.

Figure 6. The expression of GLUT4 in the soleus muscle of HFD (n=6) and HFD+Salbutamol (n=8) groups after 4 weeks of Salbutamol administration and 4 weeks after Salbutamol discontinuation.

The muscle GLUT4 expression in the HFD group did not show significant changes while its expression in the HFD+Salbutamol group was decreased by 2.74 folds ($P < 0.0001$) after salbutamol administration and by 3.15 folds ($P < 0.001$) after 4 weeks of salbutamol discontinuation (Fig. 6).

DISCUSSION

The results of this study are consistent with the reported findings that high fat diet causes insulin resistance, weight gain and the development of T2DM (19, 23, 24). The results also showed that salbutamol treatment restored insulin sensitivity, as there were no significant differences of either GTT or ITT responses of HFD+Salbutamol group compared to normal diet control group after 4 weeks of salbutamol administration, and 4 weeks after salbutamol discontinuation. E release is diminished in the elderly (20) especially during exercise (21). This could contribute to the development of the metabolic syndrome, in which insulin resistance is one of its prominent factors. Ziegler and coworkers, suggested that β_2 subsensitivity can be overcome

PGC-1 α and GLUT4 expression in the soleus muscle of HFD group

Figure 7. A. PGC-1 α and GLUT4 expression in the muscle of HFD group after 4 weeks of Salbutamol administration. **B.** PGC-1 α and GLUT4 expression in the muscle of HFD group after 4 weeks of Salbutamol discontinuation.

pharmacologically (25).

The mice used in this study were 8 months old, and at the end of the experiment they were over one year old; an age in a mouse that is comparable to a man over sixty years old (26). After 4 weeks of salbutamol treatment (HFD+Salbutamol group), the glucose profile was improved, which is consistent with the results reported by Elayan *et al.*, 2012 (19). Others suggested that β_2 stimulation would increase blood glucose (15). The acute β_2 adrenoceptor stimulation causes hyperglycemia, however in this experiment we found that chronic β_2 stimulation improved glucose tolerance.

The role of PGC-1 α expression in type 2 diabetes is still unclear and controversial. Despite that muscle PGC-1 α expression of the HFD+Salbutamol group was not significantly affected, their glucose profile was improved after 4 weeks of salbutamol administration. This improvement in blood glucose profile persisted 4 weeks after discontinuation of salbutamol in both GTT and ITT. The blood glucose profile improvement occurred despite the down regulation in muscle PGC-1 α expression in the

HFD+Salbutamol group. Moreover, after 21 weeks of HFD feeding of the HFD group, the muscle PGC-1 α was downregulated by 5.54 folds, which contributed to insulin resistance and the development of type 2 DM. These findings are consistent with the findings of Nagatomo and coworkers who demonstrated that the soleus muscles of type 2 diabetic rats had lower PGC-1 α mRNA level than those of control (23). Others reported that PGC-1 α expression was induced in mouse models of diabetes (27).

Although salbutamol did not reverse the down regulation in muscle PGC-1 α that was caused by HFD feeding in the HFD+Salbutamol group, the glucose profile was significantly lower than the HFD group, which indicates the presence of other factors in the muscle that increased glucose utilization. In support of this view, Hancock and coworkers demonstrated that overexpression of peroxisome proliferator-activated receptor delta (PPAR δ) results in a large increase in PGC-1 α protein, without the increase in PGC-1 α mRNA. They suggested that activating PPAR δ mediates a posttranscriptional increase in PGC-1 α (28).

The down regulation in PGC-1 α in the muscle of the HFD+Salbutamol group was associated with an inhibition of 3.15 folds in the expression of GLUT4 despite the improvement in the glucose profile. This unexpected result might be explained by the importance of GLUT4 translocation, rather than expression, in response to insulin. Therefore, GLUT4 translocation might be the main player in the development of type 2 DM. Interestingly, the muscle PGC-1 α expression was downregulated by a 5.54 folds in the HFD group after 21 weeks of HFD feeding without causing a significant inhibition in GLUT4 expression. Most studies however have failed to demonstrate a reduced GLUT4 expression levels in skeletal muscle of type 2 diabetic patients (7, 8).

After 17 weeks of high fat feeding, the muscle PGC-1 α expression was upregulated by 2.64 folds, and GLUT4 also showed an upregulation of 2.07 folds. However, after 21 weeks of high fat feeding, the PGC-1 α in the muscle was down regulated by 5.54 folds, and GLUT4 was downregulated by 1.35 folds. This indicates that GLUT4 follows the pattern of expression of PGC-1 α in the muscle since PGC-1 α upregulation is followed by an upregulation in GLUT4, and PGC-1 α inhibition also causes a down regulation in GLUT4. This is consistent with Michael and coworkers, who suggested that GLUT4 increased by an enhanced PGC-1 α mRNA expression (29)

The hepatic PGC-1 α expression was also tested in this study. Its level of expression was downregulated by 30.3 folds in the HFD+Salbutamol group. This was accompanied by an induction of 5.23 folds in hepatic PGC-1 α of the HFD group. These results suggest that PGC-1 α induced expression in the liver contributes to insulin resistance and decreases the liver insulin sensitivity, since PGC-1 α up regulation affects insulin sensitivity negatively in the liver (14). The mechanism by which salbutamol inhibits hepatic PGC-1 α is through β 2 receptor stimulation, since livers of most species, including humans, have a high content of both α 1 and β 2 adrenoceptors.

PGC-1 α in the liver promotes activation of gluconeogenesis and fatty acid oxidation (27), in order to synthesize glucose in the fasting state (12). However, in the fed state, the nutrient-induced rise in insulin represses gluconeogenesis and fatty acid oxidation, mainly by acetylating PGC-1 α (12). Type 2 diabetic patients have two sources of glucose in their blood in the fed state; one from the diet and another from continued glucose production from the liver (30 & 31). Hepatic PGC-1 α expression in mouse models of type 2 DM is increased (27) which is reproduced in the present study, as the hepatic PGC-1 α expression of the HFD group was up regulated by 5.23 folds.

The HFD+Salbutamol group showed a significant downregulation in PGC-1 α expression in the liver of 30.3 folds (Fig.5). This down regulation improved the glucose profile of this group and improved insulin resistance. Hepatic β 2-adrenergic receptors play an important role in the modulation of glucose production by glycogenolysis and gluconeogenesis (32).

Activation of β 2 receptors in the liver elicits hyperglycemic responses (33) and decreased hepatic β 2 activity can lead to hypoglycemia (34). However, Erraji-Benchekroun noted that the blood glucose levels measured in the transgenic mice that overexpress hepatic β 2 receptors closely resemble those observed in transgenic mice that overexpress glucokinase (GK). Increased GK expression might explain the low blood glucose concentration observed in the mice that overexpress hepatic β 2 receptors (32). Even a small increase in the expression of hepatic GK leads to a decreased blood glucose level (35).

GK activity is regulated according to fasting and refeeding (36, 37), and its transcription is decreased by glucagon through the increase in cAMP concentration (36, 38, 39). These contradictions in the role of hepatic β 2 stimulation indicate the importance of β 2 receptors

in glucose homeostasis and regulation of metabolic processes, and the effect of hepatic PGC-1 α inhibition in improving the glucose profile of diabetic patients.

After 4 weeks of salbutamol discontinuation, the blood glucose levels of the HFD+Salbutamol group were lower than those of the HFD group (Fig.3). The sustained effect of salbutamol administration, despite the four weeks of discontinuation, indicates that chronic β 2 stimulation induces a long-term improvement in glucose profile that does not decline immediately following the cessation of β 2 stimulation. In addition, the liver was taken after sacrificing the animals 4 weeks after salbutamol discontinuation, yet there was an induction in hepatic PGC-1 α of the HFD group and an inhibition in the HFD+Salbutamol group, indicating a prolonged and sustained effect of β 2 stimulation on glucose profile of diabetic mice.

In conclusion, this experiment demonstrated that chronic feeding of a high fat diet caused the development of insulin resistance and type 2 DM, along with a significant weight gain. Four weeks of salbutamol administration significantly improved insulin sensitivity and restored glucose profile towards normal. These salbutamol-induced benefits were still present four weeks after salbutamol administration was discontinued despite the continued high fat feeding. High fat feeding caused significant inhibition of the muscle PGC-1 α in both HFD and the HFD+Salbutamol groups, while it upregulated hepatic PGC-1 α in the HFD group. On the other hand, salbutamol administration caused significant down regulation in hepatic PGC-1 α , although it did not reverse the down regulation in muscle PGC-1 α caused by HFD feeding. These results were accompanied by the improvement of glucose profile and overall health of the HFD+Salbutamol group.

Conflict of interest

The authors declare that they have no conflict of interest.

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