Effects of insulin on ADPN and AdipoR1 in diabetic rats

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Summary

In this study, we have investigated insulin resistance (IR), plasma adiponection (ADPN) and ADPN receptors (AdipoR) expression in high-fat (HF) diet fed (8 weeks) rats with or without streptozotocin (STZ) treatment (30 mg/kg i.p.). Our results show that HF diet either alone or in combination with STZ treatment decreased plasma levels of ADPN and expression of muscle AdipoR. These disorders were improved with insulin (INS) treatment (1 IU/kg i.p.). In conclusion, our results suggest that ADPN might play an important role in amelioration of IR in these experimental model of type 2 diabetes.

Key words: Adiponectin, AdipoR1, insulin resistance, insulin

Introduction

The white adipose tissue has been increasingly recognized as an important endocrine organ that secretes a number of biologically active adipocytokines [1]. Adiponectin (ADPN) and Adiponectin receptor (AdipoR1) are expected to be novel preventive and therapeutic tools for control of diabetes mellitus (DM) and metabolic syndrome (MetS) [2]. ADPN mediates its effects through at least three cell membrane receptors : AdipoR1, most abundant in skeletal muscle, AdipoR2, most abundantly expressed in the liver, and recently, T-cadherin expressed on vascular endothelial and smooth muscles. These receptors mediate increased adenosine mono phosphate protein kinase (AMPK) and peroxisome proliferator-activated receptor α (PPARα) activity by ADPN binding, thus activating fatty acid (FA) oxidation and glucose (Glu) uptake [3].

Circulating ADPN level (1.9–17.0 µg/ml) in healthy adults accounting for 0.01% of total plasma proteins [4, 5]. Many factors affect plasma ADPN concentration and diurnal and pulsatile ADPN secretion has been shown in humans. ADPN peaks in the morning and decreases at night [6]. Moreover, female humans and rodents have higher plasma ADPN level than males, suggesting that sexual hormones regulate the production of ADPN [7, 8]. Some dietary factors, such as fish oils and linoleic acid [9, 10] are also suggested to increase plasma ADPN level, which is consistent with the fact that intake of these factors is thought to have a protective effect on the development of DM.

On the other hand, a carbohydrate-rich diet and high-fat diet (HF) appear to decrease plasma ADPN level [11]. Plasma ADPN concentration has also been found to be high in thin individuals, inflammation, and in Type 1 diabetes mellitus (T1DM), and low in T2DM, lipodystrophy and in obese individuals. In addition, plasma ADPN concentration is negatively correlated with body mass index [BMI), insulin resistance (IR), triglyceride (TG) and low density lipoproteins (LDL) and positively correlated with high density lipoproteins (HDL) [12].

The aim of the present study was to assess the possible contribution of ADPN in amelioration of lipide profile and IR, the metabolic abnormalities (hyperlipidemia, hyperinsulinemia and hyperglycemia) induced in diabetic, IR rats. In addition, in this study we have evaluated the effects of administration of INS on the level of ADPN, TG, total cholesterol (TC), Glu, obesity and IR index as well as the gene expression of AdipoR1 in skeletal muscle of high-fat (HF) - streptozotocin (STZ) – treated rats.

Methods

Chemicals

STZ (Trade name Zanosar) was purchased from Sigma chemical company (St. Louis Missouri, USA). The drug was dissolved in 0.1 M sodium citrate (pH 4.5). Long acting protamine zinc INS

(PZI) was purchased from Nile Company (Egypt). PZI is a pre-mixed suspension of biosynthetic human INS, 30% as soluble crystalline INS and 70% as isophan INS [13].

Experiment al Animals

For this study, 40 male Sprague-Dowley rats, 6 weeks old (145-200 g b.w.) were used. They were kept in the animal house of Kasr Al-Aini Faculty of Medicine, Cairo University. The rats had free access to food and water. They were kept at 22 ± 1ºC temperature at 12 h dark-light cycles. Animals were randomly divided into 4 groups (each, n = 10): (1) Control rats (C) were fed a standard rat chow and received an intra-peritoneal (i.p.) injection of 0.1 mol/L sodium citrate buffer (pH 4.5). The other 3 groups were fed a HF diet (35g of lard per 100g of rat chow) for 8 weeks to induce IR; (2) HF rats injected with 0.1 mol/L sodium citrate buffer , i.p., pH 4.5; (3) HF + STZ injected i.p. with a single dose of STZ (30 mg/kg) for 1 week [14]; (4) HF+STZ+INS, rats treated with INS (1 IU/kg/day) for 6 days till the end of the study protocol (8 weeks). DM was verified after 5 days by measuring blood Glu levels (after overnight fast) with the use of Glu oxidase reagent strips (Lif3 scan, Milpitas, CA, USA). Rats having blood Glu level $\geq$200 mg/dl, were considered diabetic [15]. After the end of 8 weeks animals retro-orbital blood samples were obtained under anesthesia using pentobarbital 40 mg/kg, b.w. i.p., after overnight fasting. Animals were weighted and the naso-anal length was measured in cm [16]. The blood samples were withdrawn through the retro-orbital route using heparinized capillary tubes. The blood samples were allowed to clot for 20 min and then centrifuged at 10,000 rpm for 20 minutes for plasma separation and stored at -70°C until time of assay of plasma levels of ADPN, Glu, INS, TG and TC. The animals were sacrificed by blow to the head and tissue samples from soleus muscle (30 mg) were dissected and kept frozen at –80°C in liquid nitrogen until they were used to assess the AdipoR1 gene expression.

The experiments were conducted in accordance with Ethical Guidelines for investigations of laboratory animals and were approved by the Ethical Committee of Kasr Al-Aini faculty of Medicine , Cairo University.

Measurements of Biochemical Parameters

Measurement of fasting plasma INS

Rat INS concentration was measured in plasma by enzyme immunoassay using the rat INS ELISA kit (Linco Research, MO). The assay was performed according to the standard procedure of the ELISA kits. Absorbance was measured at 450 nm.

Measurement of fasting plasma Glu

The plasma Glu was assayed by the method adopted by Trinder [17]. The test materials for this method were supplied as kits by "Diamond Diagnostics". Absorbance was measured at 500 nm .

Measurement of obesity index (OI)

The OI (the equivalent of body mass index in humans) was calculated according to an equation formulated by Dubuis [18]:

$$OI = \sqrt[3]{weight(gm)} \text{ x 1000/naso-anal length (cm)}$$

Homeostasis model assessment of IR (HOMA-IR)

The assessment of IR was devised by Matthews [19] and it depends on relationship between fasting plasma Glu and INS based on a mathematical model: HOMA-IR = {[Fasting Glu (mg/dl)/18) X Fasting INS (µIU/ml)}/ 22.5

Measurement of fasting plasma TG

Fasting plasma TG was assayed by the method adopted by Wahlefeld [20]. TG quantification kit was used to supply the test materials for this method (BioVision Research). Absorbance was measured at 570 nm.

Measurement of fasting plasma TC

Fasting plasma TC was assayed by the method adopted by Sundvall [21]. Cholesterol assay kit was used to supply the test materials for this method (BioAssay Systems)**.** Absorbance was measured at 340 nm.

Measurement of fasting plasma ADPN

Serum level of ADPN was determined by using rat ADPN ELISA kit (Linco Research, MO) according to the manufacturer's instruction. Absorbance was measured at 450 nm.

Detection of AdipoR1 gene expression by polymerase chain reaction (PCR) in the soleus muscle

For the detection of AdipoR1 gene expression, RNA was extracted was from soleu muscle homogenate using SV-Total RNA isolation system kit (Promega Biosciences, Medison, USA) according to the manufacturer's instruction and the extracted RNA was measured spectrophotometrically at 280 nm. Extracted RNA was reverse transcribed into cDNA, and amplified

by PCR according to Williams [22]. SV-Total RNA kit was used to supply the test materials. The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene USA). cDNA was prepared from RNA as follows: about, 20µg of mRNA was heated at 70 ºC for 5 min with 50 pmol of reverse primer of AdipoR1 gene before adding 5 X RT buffer 50 mM Tris-HCL pH 8.3. 10 mM dNTPS and 200 U of moloney, MO murine leukemia virus reverse transcriptase in a final volume up to 36µL was added. RT reaction was carried for 2 h at 37 ºC.

Polymerase Chain Reaction (PCR)

5 µL of cDNA was subjected to PCR under the conditions specified below; PCR reaction was carried by adding 50 pmol of each of forward and reverse primer specific to AdipoR1 gene. The oligonucleotide primers sequences of AdipoR1 gene are presented in Table 1. For each sample, the master mix contained : 5 µl of 10 x PCR buffer, 1 µl of 10 mM dNTPs, 2 primers (50 pmol for each), 1 µl of Taq polymerase, and 37 µl of distilled water. The total volume of each sample master mix was 45 µl. 5 µl of cDNA was pipetted and added to the master mix. The tube was inserted in the thermal cycler and the cycling conditions are shown in **Table 1**.

TABLE 1. Primer design and PCR protocols (annealing temperature and number of cycles). AdipoR1 = adiponectin receptor 1

Forward primer:	Cycling conditions	
5′-AAT GTTTCAGTGCAGAG-3′	95°C → 1 min → Denaturation	
Reverse primer:	55°C → 1 min → Annealing	35 cycles
5′-TTG GGATGATGTCGGGAC-3′	72°C → 2 min → Elongation	

Agarose Gel Electrophoresis

The amplified PCR product of AdipoR1 gene were electrophoresed on 1.5 % Agarose gel and UV visualized after staining with ethidium bromide. A densitometry system using a Standard DNA of known concentration gene Gel, documentation system was used for analysis (Syngene, Cambridge, UK). PCR products were semiquantitated by using gel documentation system (Biometra Germany).

Statistical Analysis

Data were processed using the statistical package SPSS version 12. The results are presented as mean ± SD. Comparisons were made by unpaired *t* - test or one-way ANOVA as required. When a significant F was obtained, multiple comparisons post tests were used to determine which groups were significantly

different. Linear correlation or regression was also assessed between different variables using the Pearson product-moment correlation coefficient. P ≤ 0.05 was considered significant.

Results

First, we have shown (**Figure 1**) that OI in HF and HF+STZ rats were significantly increased when compared to C group (C = 317±12.8, HF = 340±3.9, HF+STZ = 329±4.68, P<0.001) . We further questioned whether the TG and TC, Glu, INS and HOMA index, ADPN level were significantly changed in HF and HF + STZ rats compared to C.
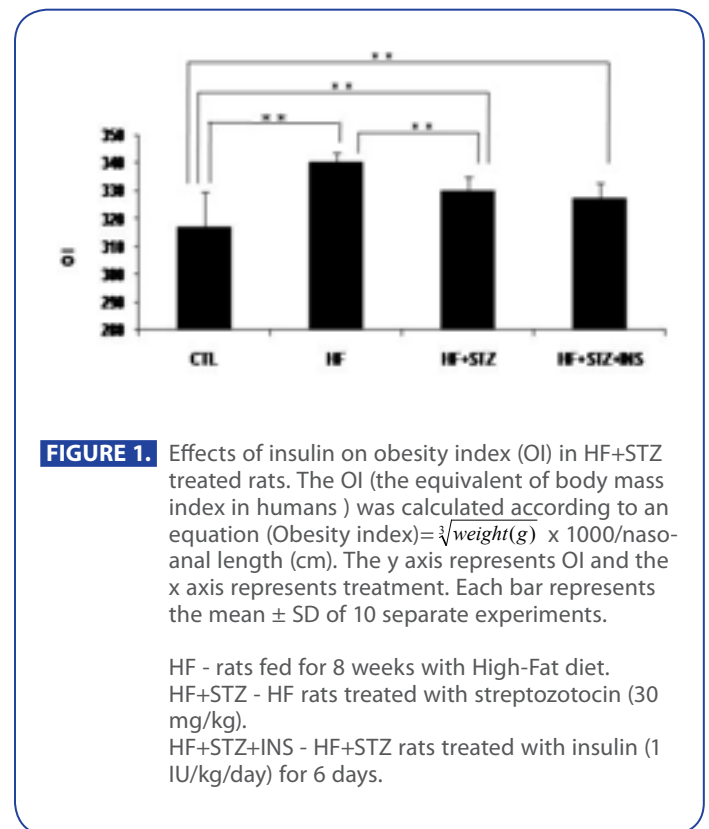

FIGURE 1. Effects of insulin on obesity index (OI) in HF+STZ treated rats. The OI (the equivalent of body mass index in humans) was calculated according to an equation (Obesity index)= $\sqrt[3]{weight(g)}$ x 1000/naso-anal length (cm). The y axis represents OI and the x axis represents treatment. Each bar represents the mean ± SD of 10 separate experiments.

HF - rats fed for 8 weeks with High-Fat diet.
HF+STZ - HF rats treated with streptozotocin (30 mg/kg).
HF+STZ+INS - HF+STZ rats treated with insulin (1 IU/kg/day) for 6 days.

Figure 2 shows that plasma level of TG and TC were significantly increased in HF and HF + STZ rats compared to C (TG: C = 57.35 ± 6.0 mg/dl; HF = 74.88±7.1mg/dl; HF+STZ = 85.55±5.7mg/dl; TC: C =127.37 ±5.5 mg/dl; HF =157.95±10.8mg/dl; HF+STZ = 173.55 ± 11.7mg/dl, P<0.001). To test whether the increase in TG and TC was the result of a increase in Glu, INS and decrease in adiponectin level we analyzed the level of Glu, INS and adiponectin in C, HF and HF+STZ animals. HF diet and STZ treatment of HF rats significantly increased plasma Glu and INS level compared to C (Glu: C = 79.665 ± 3.7 mg/dl; HF = 141.21
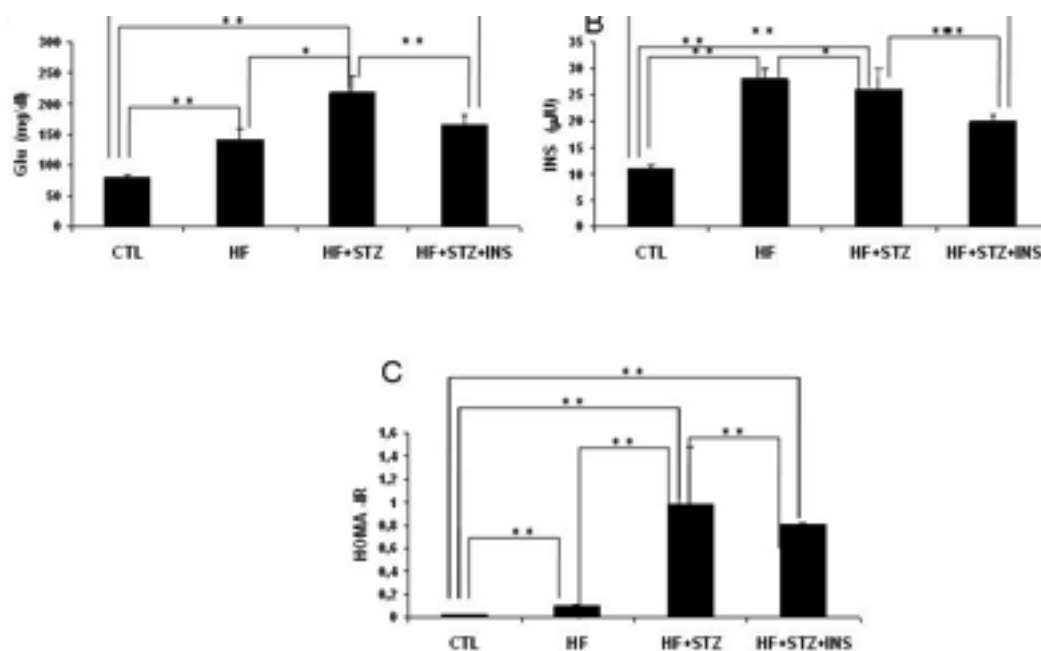
FIGURE 2. Total cholesterol (TC) and triglycerides level (TG) in HF+STZ – insulin treated rats. Fasting plasma TC **(A)** and fasting plasma TG **(B)** of HF, HF+STZ and HF+STZ+INS rats were determined as described in "Methods" section. The y axis represents plasma level of TC (A) and TG **(B)** expressed as mg/dl and the x axis represents treatment. Each bar represents the mean±SD of 10 separate experiments The meanings of other abbreviations as the same as in Figure 1.
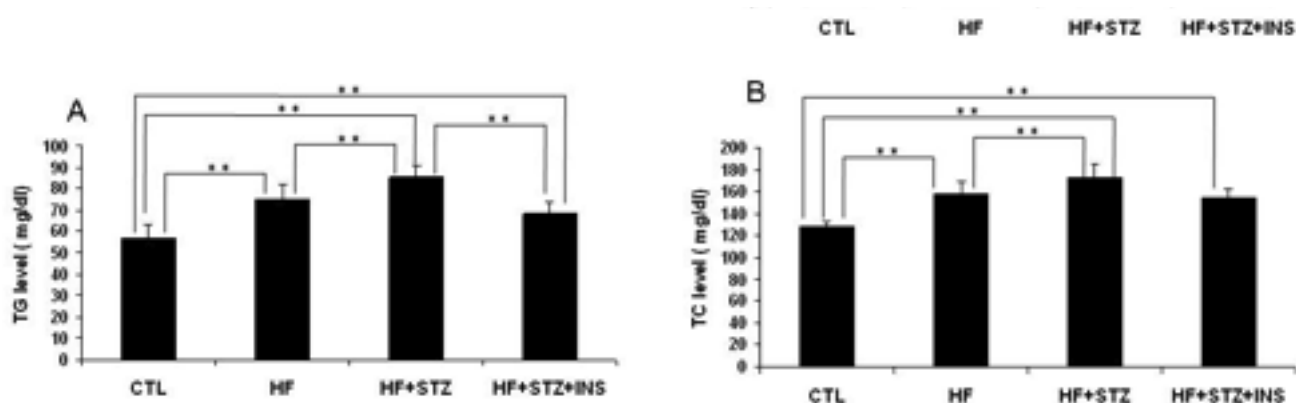

FIGURE 3. Insulin (INS), glucose (Glu) level and insulin resistance (HOMA-index) in C, HF, HF+STZ and HF+STZ+INS treated rats. **(A)** Rat INS concentrations were measured in plasma by enzyme immunoassay using the rat INS ELISA kit. The y axis represents INS plasma level expressed as 0IU/L and the x axis represents treatment. **(B)** The plasma Glu level was assayed as described in "Methods" section. The y axis represents Glu plasma level expressed as mg/dl and the x axis represents treatment. **(C)** The assessment of IR was depends on relationship between fasting plasma Glu and INS based on a mathematical model: HOMA-IR = {[Fasting Glu (mg/dl)/18] X Fasting INS (µIU/ml)}/ 22.5. The y axis represents HOMA-IR and the x axis represents treatment. Each bar represents the mean±SD of 10 separate experiments. The meanings of other abbreviations as the same as in Figure 1.
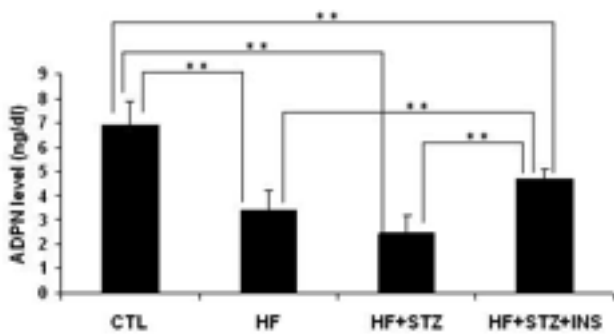
FIGURE 4. Level of Adiponectin (ADPN) in serum of C, HF, HF+STZ and HF+STZ+INS rats. Serum of ADPN was determined by using rat ADPN ELISA kit as described in "Methods" section. The y axis represents ADPN level expressed as ng/dL and the x axis represents treatment. Each bar represents the mean±SD of 10 separate experiments. The meanings of other abbreviations as the same as in Figure 1.
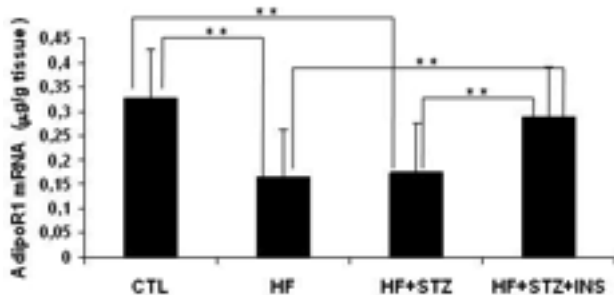

FIGURE 5. Adiponectin receptor 1 (AdipoR1) gene expression in skeletal muscle of C, HF, HF+STZ and HF+STZ+INS rats. Expression of gene for AdipoR1 in skeletal muscle was measured by RT-PCR method as described in "Methods" section. The y axis represents AdipoR1 expressed as 0g/g tissues and the x axis represents treatment. Each bar represents the mean±SD of 10 separate experiments. The meanings of other abbreviations as the same as in Figure 1.

± 18.5mg/dl; HF+STZ = 219.9 ± 26.87mg/dl, P<0.001; INS: C = 11.05 ± 0.70IU; HF = 28.517 ± 2.30IU; HF+STZ =36.65±4.00IU, P<0.001).

In addition, ADPN level was significantly decreased in in HF and HF+STZ rats compared to C (C = 6.888 ± 1.0 ng/dl; HF = 3.347±0.9 ng/dl; HF+STZ = 2.45±0.7 ng/dl, P<0.001). This de-

creases in ADPN level was followed by significantly decreses in AdipoR1 expression in HF and HF + STZ rats compared to C (C = 0.326 ± 0.10g/g tissue ; HF = 0.163±0.10g/g tissue; HF + STZ = 0.175 ± 0.10g/g tissue , P<0.001). Furthermore, treatment with INS significantly (P<0.001) decresed plasma level of Glu (Fig. 2A), INS level (Fig. 3B) and IR (Fig. 3C). Treatment with INS also decreased plasma level of TG (Fig.2B) when compared to HF+STZ rats.

In addition, INS also partially restored the plasma ADPN (**Fig. 4**) and gene expression of AdipR1 (**Fig.5**) as compared to the HF + STZ group, but it did not go back to the control level.

Correlation study, show that plasma ADPN and AdipoR1 were significantly (P<0.05) negatively correlated with the following parameters: OI, TC, TG, INS, Glu level and HOMA-IR. In addition, there is also a significant positive correlation between AdipoR1 and ADPN.

Discussion

In this study we have shown that HF diet for 8 weeks caused significant metabolic changes, as compared to the control rats which were fed on the standard laboratory chow. These metabolic changes included increase in OI, hyperlipidemia, hyperinsulinemia and hyperglycemia which finally induced increased IR. In addition, these metabolic changes were accompanied by hypoadiponectinemia and down regulation of AdipoR1 expression in skeletal muscle.

Work done by Bluher [23] hypothesized that in the steady state, ADPN level would be lower, and its receptors level would be higher in obese people as compared to control subjects to compensate for the low AdipoR1 level observed in subjects with obesity and IR. Moreover, plasma ADPN level is correlated inversely to mRNA expression of both AdipoR1 and AdipoR2 so the lower the ADPN plasma level, the higher would be the expression of the receptors. Though, in this study we have found a negative correlation between the increased levels of TC, TG and OI with ADPN and Adipo R1 expression in skeletal muscle of HF rats but our results show a positive correlation between plasma ADPN and AdipoR1 expression. However, our experiment only lasted for 8 weeks, so it is possible that rats did not still reach the steady state. Our results are also differ from the results reported by Barnea [24] who documented that AdipoR1 expression increased in muscle tissue, while plasma ADPN level did not change in HF rats for 4 months, however, they used different rat strains and a longer duration of experiment and a different composition. Thus, changes in AdipoR1 mRNA levels do not always reflect a parallel change in plasma total ADPN level [25].

INS treatment is among the alternative strategies designed to manage T2DM and it results in improvement of glycemic control and INS synthesis and secretion by β-cells [26]. INS therapy

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decreases hepatic Glu output and improves peripheral Glu uptake and may also improve IR [27]. In our study, INS treatment resulted in an improvement in IR as manifested by decreasing the fasting plasma lipids, INS and Glu levels as compared to untreated diabetic group. INS also significantly increased both ADPN and AdipoR1 back to the control values. This effect mimicks the effect of INS therapy in humans which was demonstrated by Langouche [28] who postulated that INS therapy increased circulating ADPN level, normalized the elevated serum C-peptide level and decreased IR by increasing the metabolic INS signal in human muscle. It has been shown that administration of INS to T2DM rats noticeably improved the INS content of β-cells, with a slight reduction in fasting blood Glu and TG as compared to an untreated diabetic (HF + STZ) group [26].

Despite the significant reduction in Glu level and improvement of IR in INS-treated diabetic (HF + STZ + INS) rats as compared to the untreated diabetic (HF + STZ) rats in our study, the INS treatment partially restore the Glu level in comparison to the control group. This results may be explained by work of Mason [29] who reported that INS treatment by i.p. route, normalized the elevated Glu production in STZ-diabetic rats, while INS delivered by SC route only partially normalized it. In addition, this also may be due to our different dose of INS (once instead of twice/day) and due the short duration of treatment before the measurements were done (6 days).

Conclusion

Results from this study demonstrated that feeding rats with HF-diet for 8 weeks in addition to a low dose of STZ, induced metabolic abnormalities and increased IR. It was also associated with hypoadiponectinemia and down regulation of AdipoR1 expression in skeletal muscle. All these metabolic changes were significantly improved by administration of INS which also resulted in increasing both plasma levels of ADPN and AdipoR1 expression in skeletal muscle. These results suggest that ADPN hormone might play an important role in the amelioration of the lipid profile and IR that is observed with INS treatment in pathophysiological conditions such is diabetes.

Acknowlegments

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