ADIPONECTIN REGULATION IN TYPE 2 DIABETIC RATS: EFFECTS OF INSULIN, METFORMIN AND DEXAMETHASONE

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ABSTRACT

Adiponectin is a protein synthesized from adipose tissue, increases peripheral glucose utilization in liver and skeletal muscle. Adiponectin expression and secretion are decreased during obesity and insulin resistance. In this study, the effect of insulin, metformin and dexamethasone on serum lipid profiles was examined in Type 2 Diabetic (T2D) rats. T2D was induced by feeding rats a high fat diet for 4 weeks plus medium dose of Streptozotocin (STZ, 35 mg kg$^{-1}$ BW). Adiponectin, adiponectin receptors (AdipoR-1 and AdipoR-2), leptin, Peroxisome Proliferator Activated Receptor gamma (PPAR-$\gamma$), Hormone Sensitive Lipase (HSL), Pyruvate Kinase (PK), enolase and Glucose Transporter-2 (GLUT-2) expression in epididymal adipose and liver tissue were examined using RT-PCR. Results showed that metformin improved insulin resistance by normalizing serum lipid profiles in diabetic rats, while dexamethasone did not alter it. Metformin up-regulated adiponectin, AdipoR-1 and AdipoR-2 expression, while insulin and dexamethasone down-regulated them. Leptin expression was decreased while PPAR$\gamma$, HSL, PK, enolase and GLUT-2 expression was increased by metformin administration. Dexamethasone failed to improve insulin resistance in T2D rats. In conclusion, metformin ameliorates T2D through controlling adiponectin expression and its consequent genes of lipids and glucose metabolism.

Keywords: Adiponectin Regulation, Diabetes, Metformin, Insulin, Glucose and Lipid Metabolism

1. INTRODUCTION

Improvements in technology lead to a sedentary lifestyle and consequently, an increase in body weight and obesity (Goldstein and Scalia, 2004). T2D is a quickly growing global metabolic disease characterized by impaired insulin secretion from pancreatic $\beta$ cells and insulin resistance in liver, muscle and adipose tissue (Ashcroft and Rorsman, 2012). Insulin resistance is closely linked with other metabolic symptoms such as hypertension and hyperlipidemia (Cornier et al., 2008). Insulin resistance is frequently associated with obesity, however, the molecular link between increased adiposity and reduced sensitivity of target tissues to insulin is not completely clear (Kahn and Flier, 2000; Winter et al., 2006).

Kwon and Pessin (2013) reviewed that adipose tissue was considered as an inert energy storage organ that accumulates and stores triacylglycerols during energy excess and releases fatty acids in times of systemic energy need. However, over the last two decades adipose tissue depots have been established as highly active endocrine and metabolically important organs that modulate energy expenditure and glucose homeostasis. White adipose tissue is responsible for the secretion of an array of signaling...
Adiponectin is a protein which was originally identified by Scherer et al. (1995). It is encoded by the Adipo Q gene in humans and has recently attracted much more attention (Capeau, 2007; Kwon and Pessin, 2013). Adiponectin is a member of the soluble defense collagen superfamily and plasma adiponectin concentrations and mRNA expression have been shown to be decreased in obesity and insulin resistance (Brun et al., 2003; Weyer et al., 2001). Its primary structure contains an N-terminal signal sequence, a variable domain, a collagen-like domain and a C-terminal globular domain (Yamauchi et al., 2003). Adiponectin stimulates fatty acid oxidation in liver and muscle, decreases plasma triglycerides and enhances glucose metabolism by increasing insulin sensitivity (Combs et al., 2001; Kloting et al., 2010; Yamauchi et al., 2001). Adiponectin is involved in the regulation of energy balance and body weight (Fruebis et al., 2001; Yamauchi et al., 2001). Its synthesis and secretion are regulated by several factors including IGF-1 and insulin (Halleux et al., 2001) and are affected by thiazolidinediones (Bodles et al., 2006). Diez and Iglesias (2003) suggested that plasma level of adiponectin has been reduced in clinical conditions associated with insulin resistance, including obesity, type 2 diabetes, dyslipidemia and hypertension. Moreover, it has been reported that high molecular weight form of adiponectin may serve as a significant surrogate marker for the prediction of type 2 diabetes in Japanese population (Sato et al., 2010). Adiponectin has two adiponectin receptors, adipoR1 is the major receptor expressed in skeletal muscle, whereas adipoR2 is mainly expressed in liver (de Oliveira et al., 2011a). Both receptors are expressed in various tissues and pancreatic cells (Beylot et al., 2006).

Treatment of type 2 diabetes depends on oral hypoglycemic drugs that contain Peroxisome Proliferator Activated Receptor Gamma (PPARγ) and thiazolidinediones, among which metformin is the most common. These drugs act on the improvement of hyperinsulinemia, an integral part in the development of type 2 diabetes (Gerich, 1998). Metformin is widely used as a first line of treatment for patients with type 2 diabetes mellitus. Metformin improves glucose metabolism mainly by suppressing hepatic glucose production (Hundal et al., 2000), but its exact mechanism of action remains unclear. Several reports have concluded that metformin does not significantly affect plasma adiponectin levels (Tiikkainen et al., 2004).

Adiponectin is regulated by several hormones and factors in humans, rodents and cattle (Birkenfeld et al., 2012; de Oliveira et al., 2011a; Fasshauer et al., 2002; Soliman et al., 2011). Catecholamines (Fu et al., 2007), growth hormones (Xu et al., 2004), insulin (Fasshauer et al., 2004) and glucocorticoids (Fasshauer et al., 2001) are mostly examined. Both in vitro and in vivo studies have shown that glucocorticoids reduce plasma levels of adiponectin and inhibit adiponectin expression (Jang et al., 2008). Moreover, Shi et al. (2010) observed decreased serum levels of adiponectin and reduced adiponectin mRNA expression in the adipose tissue of both obese and non-obese rats treated with glucocorticoids. Relatively few studies have analyzed the effects of glucocorticoids on the expression of adiponectin and its receptors during insulin resistance. Among glucocorticoids, dexamethasone is a synthetic glucocorticoids frequently used to rapidly generate insulin resistance in rodents (Besse et al., 2005). In this study, we examined the effects of metformin, insulin and dexamethasone on insulin resistance with regard to adiponectin and gene expression of lipids and glucose.

2. MATERIALS AND METHODS

2.1. Materials

Streptozotocin (STZ), insulin, metformin, dexamethasone were purchased from sigma Aldrich, USA. The Wistar albino rats were purchased from Egyptian Co for Experimental Animals Import, Helwan, Cairo, Egypt. Solvents and related materials were from ADWIA pharmaceutical company, Egypt. High fat diet was purchased from Qaha Co. Qaha, Kalubiya, Egypt. Biochemical kits for lipids profiles were from Clini Lab, Cairo, Egypt.

2.2. Induction of Type 2 Diabetes (Insulin Resistance) and Experimental Design

Male Wistar rats (50 rats), 4 weeks old, weighting 80-100 g, were selected randomly. Rats were exposed to 12 h/12h day light with free access to food and water.
The fifty rats were divided into five groups (10 rats per group). Control group was fed normal diet and the remaining 4 groups Fed High Fat Diet (HFD) for one month. The HFD constitutes 15.5% protein, 38.8% fat and 45.7% carbohydrates, by calories. Induction of T2D in HFD rats was based on the protocol of Srinivasan et al. (2005) (intraperitoneal injection of medium dose of STZ, 35 mg kg\(^{-1}\) BW together with HFD). Diabetes was confirmed after 3 days, as serum glucose and lipid profiles were measured and values of glucose over 200 mg dL\(^{-1}\) considered diabetic. All diabetic rats (n = 40) were subdivided into 4 subgroups, diabetic group (n = 10), diabetic plus insulin (n = 10) received insulin subcutaneously in a dose of 0.2 units/kg/day for 14 days, diabetic plus metformin (400 mg kg day\(^{-1}\)) for 14 days and diabetic plus dexamethasone (0.2 mg 100\(^{-1}\) g BW twice per day S/C) for 14 days.

After the end of experimental procedures, all rats were decapitated after overnight fasting and blood was collected for serum extraction. Liver and adipose tissues were preserved in TriZol for RNA extraction and gene expression.

2.3. Serum Chemistry Analysis

Serum Triglycerides (TG), Total Cholesterol (TC), Low Density Lipoproteins (VLDL), LDL and High Density Lipoproteins (HDL) were measured using commercial kits that based on spectrophotometric analysis.

2.4. RT-PCR Analysis and Gene Expression

Liver and epididymal adipose tissues were collected from rats, flash frozen in 1 mL TriZol reagent (Invitrogen, Carlsbad, CA) and subsequently stored at -70°C. Frozen samples (approximately 100 mg of tissue per sample) were immediately homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Total RNA was extracted via chloroform extraction followed by nucleic acid precipitation with isopropanol. The pellet was washed with 75% ethanol and resuspended in molecular biology grade water. Nucleic acid concentration was measured using o.d. 260 nm (Smart-Spec; Bio-Rad Laboratories, Hercules, CA) and RNA integrity was evaluated using an Agilent bioanalyzer (model 2100; Agilent Technologies, Foster City, CA).

RNA (1 µg) was treated at 70°C for 5 min and reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco), 50 pmol of poly (dT) primer and 20 nmol of dNTPs in a total volume of 10 µL at 37°C for 1 h. After heating at 94°C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (Perkin-Elmer, Foster City, CA, USA), 3 mM MgCl2 and 50 pmol of forward and reverse primers specific for respective genes in a total volume of 25 µL. The PCR conditions of different tested genes are shown in Table 1. PCR product was visualized under UV lamp by electrophoresis in 1.5% agarose gel stained with ethidium bromide. Intensities of PCR bands will be analyzed densitometrically using NIH Image program (http://rsb.info.nih.gov/nih-image/).

2.5. Statistical Analysis

Results are expressed as means±S.E. for 5 independent rats per each group. Statistical analysis was done using ANOVA and Fischer’s post hoc test, with p<0.05 being considered as statistically significant.

| mRNA expression | Forward primer | Reverse primer | PCR cycles and Annealing Temp |
|------------------|----------------|----------------|-------------------------------|
| PK (229 bp)      | 5'-ATGCTGTGACTGGATCTGC-3' | 5'-CCCCCATGATGTTGAGATTAG-3' | 30 cycles, 52°C for 1 min |
| PEPCK (236 bp)   | 5'-TTACTGGGAAGCGATGAT-3' | 5'-TCGTAGACAAGGGGACAC-3' | 30 cycles, 52°C for 1 min |
| GLUT-2 (330 bp)  | 5'-AAGGATCAAGCGATGATGAGG-3' | 5'-GGACCTTCTGCTCAGTGGG-3' | 30 cycles, 55°C 1 min |
| LPL (269 bp)     | 5'-CCTGTGACTGGATGTTGATG-3' | 5'-AGGCAAGCGGCCGCTGAGATCTC-3' | 24 cycles, 60°C for 45 sec |
| Leptin (244bp)   | 5'-CTTGTTGCTGGTCTGCTAGTCC-3' | 5'-ATGCTGTTGCTGGTGTTTTC-3' | 35 cycles, 61°C for 1 min |
| PPAR-γ (550 bp)  | 5'-CATTCTCTGCTCCAACATGAA-3' | 5'-CGGGAAGGACTTTATGATGAG-3' | 35 cycles 51°C for 1 min |
| Adipo (500 bp)   | 5'-CTCAGCAAGGGAAACTTGT-3' | 5'-CTGTGCTTCTTTTCTCAGT-3' | 35 cycles, 59°C for 1 min |
| Adipo-1 (398bp)  | 5'-AAGGAGATGAGCTGGAGA-3' | 5'-CCTGTTCCAGCTCCTACG-3' | 37 cycles, 58°C for 1 min |
| Adipo-2 (233bp)  | 5'-GCCAGAGGACTTCTGAGCTG-3' | 5'-GACTGAGCCGACGATTACC-3' | 37 cycles, 58°C for 1 min |
| HSL (301 bp)     | 5'-GCCAGAGGACTTCTGAGCTG-3' | 5'-AGGACACCTTCTGAGCCG-3' | 40 cycles, 61°C for 1 min |
| GAPDH (309 bp)   | 5'-AGATCCCAACGCCGATCATT-3 | 5'-TCTCCCAAGATGTTGACAA-3' | 25 cycles, 52°C 1 min |

PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle were 94°C, 30 sec and 72°C, 60 sec, respectively.
3. RESULTS

3.1. Effects of Insulin, Metformin and Dexamethasone on Lipid Profiles in Diabetic Rats

As shown in Table 2, induction of diabetes in rats increased serum cholesterol, TG, VLDL and LDL levels and decreased HDL levels. Administration of insulin and metformin significantly improved such amelioration in lipid profiles compared to control and diabetic rats. Dexamethasone administration failed to improve such changes in lipid profiles compared to diabetic rats.

3.2. Effects of Insulin, Metformin and Dexamethasone on Insulin and Glucose Levels in Diabetic Rats

To test the effect of adiponectin regulators on insulin and glucose levels in type 2 diabetic rats, Fig. 1 showed that feeding of high fat diet for 1 month together with small dose of STZ induced insulin resistance. Insulin resistance was characterized by increase in insulin and glucose levels. Insulin administration increased insulin levels in blood of diabetic rats but partially lower glucose concentrations. While metformin administration clearly improved insulin resistance, decreased insulin and consequently glucose concentrations (Fig. 1). Dexamethasone is known as glucogenic glucocorticoid that increases glucose levels. Dexamethasone failed to normalize or improve insulin resistance seen in diabetic rats (Table 2 and Fig. 1).

3.3. Effects of Insulin, Metformin and Dexamethasone on Adiponectin and Adiponectin Receptor Expression in Epididymal Adipose Tissue

Diabetic rats showed a decrease in adiponectin expression relative to control (Fig. 2A) and insulin administration induced additive inhibitory effect on adiponectin expression, while metformin induced a significant and clear up-regulation in adiponectin expression. Dexamethasone administration induced inhibition in adiponectin expression. Regarding adiponectin receptor-1 expression (AdipoR-1), Fig. 2B showed that AdipoR-1 expression was down regulated in diabetic rats and insulin administration failed to recover this inhibition, while metformin up-regulate it. Dexamethasone partially normalized AdipoR-1 expression compared to control and diabetic rats. Next, we tested AdipoR-2 expression, as seen in Fig. 2C, AdipoR-2 expression was decreased in diabetic and insulin administered rats. AdipoR-2 expression increased by metformin and decreased by dexamethasone.

Table 2. Changes in lipid profiles after administration of insulin, metformin and dexamethasone into type 2 diabetic Wistar rats

|                  | Control      | Diabetic group | Diabetic + Insulin | Diabetic + Metformin | Diabetic + Dex |
|------------------|--------------|----------------|-------------------|----------------------|---------------|
| Cholesterol      | 118.6±2.9    | 190±4.4        | 107.3±4.9#        | 103.6±3.5#          | 201.6±8.1     |
| TG               | 98.3±6       | 195.7±10.5*    | 100.5±2.9#       | 80.6±5.2#           | 152±10.2      |
| LDL              | 57.6±4.4     | 208.6±9.6*     | 55.6±5.2#        | 58.6±1.8#           | 149.3±1.8     |
| VLDL             | 19.6±2.1     | 38±2.1*        | 20.6±0.9#        | 17.3±1.8#           | 43.6±1.3      |
| HDL              | 34±1         | 23.6±2.1       | 33.3±0.8#        | 32.8±1.2*           | 24±3.2        |

Data are presented as (means ± S.E.). S.E = Standard error for 5 different rats per each group. *p<0.05 Vs control and #p<0.05 Vs diabetic rats

Fig. 1. Serum changes in insulin and glucose after administration of insulin, metformin and dexamethasone into type 2 diabetic Wistar rats. Results are expressed as means ± SE for 5 different rats. Insulin and glucose was measured using commercial kits.
Fig. 2. RT-PCR analysis of adiponectin, AdipoR-1 and AdipoR-2 expression in epididymal adipose tissue after administration of insulin, metformin and dexamethasone into type 2 diabetic Wistar rats. RNA was extracted and reverse transcribed (1 µg) and RT-PCR analysis was carried out for adiponectin, AdipoR-1 and AdipoR-2 expression as described in materials and methods.
Fig. 3. RT-PCR analysis of leptin, PPAR-γ and HSL expression in epididymal adipose tissue after administration of insulin, metformin and dexamethasone into type 2 diabetic Wistar rats. RNA was extracted and reverse transcribed (1 µg) and RT-PCR analysis was carried out for leptin, PPAR-γ and HSL expression as described in materials and methods.
Fig. 4. RT-PCR analysis of PK, enolase and GLUT-2 expression in liver tissue after administration of insulin, metformin and dexamethasone into type 2 diabetic Wistar rats. RNA was extracted and reverse transcribed (1 µg) and RT-PCR analysis was carried out for PK, enolase and GLUT-2 expression as described in materials and methods.
3.4. Effects of Insulin, Metformin and Dexamethasone on Lipolytic Gene Expression in Epididymal Adipose Tissue

In type 2 diabetic rats, the expression of leptin, PPAR-γ and HSL were not changed (Fig. 3). Insulin administration induced significant increase in leptin and HSL expression without changes in PPAR-γ. Metformin induced significant increase in expression of PPAR-γ and HSL and a decrease in the expression of leptin (Fig. 3). While, dexamethasone induced upregulation in leptin expression without changes in PPAR-γ and HSL expression.

3.5. Effects of Insulin, Metformin and Dexamethasone on PK, Enolase and GLUT-2 Gene Expression in Liver Tissue

In diabetic rats PK, enolase and Glucose Transporter-2 (GLUT-2) expression was decreased (Fig. 4). Insulin administration increased PK and enolase expression in a way to improve insulin resistance. Moreover, metformin administration improved the decrease in glycolysis recorded in diabetic rats and increased the expression of PK and enolase. Dexamethasone inhibited expression of PK and enolase and failed to improve insulin resistance in diabetic rats. As seen in Fig. 4C, diabetes inhibited GLUT-2 expression and administration of insulin and metformin up-regulate (1/2 fold for insulin and 1.5 fold for metformin respectively), while, dexamethasone down regulate GLUT-2 expression in liver of diabetic rats.

4. DISCUSSION

Results of this study showed the importance of adiponectin in regulation of T2D. Administration of metformin and to less extent insulin improved insulin sensitivity (Table 2) and decreased glucose levels in blood (Fig. 1). Metformin and/or troglitazone are commonly used in treatment of T2D and mediate their insulin sensitizing actions via distinct metabolic pathways (Ciaraldi et al., 2002). In adipocytes, troglitazone, a member of the thiazolidinediones (TZD), seems to enhance anabolic pathways, leading to an increase in adipocyte size, while metformin appears to stimulate catabolic pathways, which results in a reduced adipocyte size (Ciaraldi et al., 2002). It is possible that both metformin and troglitazone cause differences in production of adiponectin. Adiponectin is reduced in type 2 diabetes (Weyer et al., 2001). Moreover, metformin increased adiponectin gene expression and circulating hormone levels (Phillips et al., 2003). Our results showed that metformin (PPAR-γ agonist) increased adiponectin and its receptors expression in T2D rats. Thus the insulin-sensitizing effect of troglitazone appears to be mediated through an elevation in adiponectin expression (Yu et al., 2002). It was suggested that modulation of adipocytes function including upregulation of adiponectin synthesis and secretion, is an important mechanism by which thiazolidinediones improve insulin action (Phillips et al., 2003; Tiikkainen et al., 2004; Yu et al., 2002). Our findings confirmed that metformin is a more efficient in improving insulin sensitivity and T2D. This is supported by the findings of Bailey and Day (2004) and Fonseca et al. (2000).

Hyperglycemia promoted by the high-fat diet with medium dose of STZ was accompanied by a reduction in adiponectin, Adipor1 and Adipor2 gene expression (Fig. 2) in the epididymal adipose tissue, suggesting the involvement of adiponectin in T2D incidence. Other studies have suggested that increased insulin levels due to a high-fat diet may affect expression of the adiponectin receptors (Bullen et al., 2007; Liu et al., 2007). Our findings supported this idea as insulin decreased adiponectin and Adipor1 expression in adipose tissue. It is well established that increased insulin levels due to a high-fat diet and medium dose STZ affected the expression of the adiponectin receptors (Bullen et al., 2007; Liu et al., 2007). Insulin represses the expression of both adiponectin receptors in muscle and liver (Liu et al., 2007), that adipor1, but not adipor2, is repressed by insulin (Inukai et al., 2005). Therefore and possibly because insulin levels were high in T2D than control group, we can assume that the changes in the expression of adiponectin and its receptors is due to the increase in insulin levels.

Insulin resistance induced by glucocorticoids is associated with a decrease and an alteration in adiponectin receptors and consequently is the main cause of hyperglycemia (de Oliveira et al., 2011b) and that are consistent with our findings (Table 2 and Fig. 2). Serum adiponectin levels are negatively associated with the BMI in healthy individuals and are decreased in T2D (Margoni et al., 2011). Therefore, adiponectin may be a local regulator for glucose utilization in the adipocytes and adipose tissue via its regulation of PPAR-γ, glucose and lipids transcriptional factors expression (Ajuwon and Spurlock, 2005; Yamauchi et al., 2001).

Unlike adiponectin, metformin inhibited leptin expression while insulin up-regulate it. Therefore, metformin suppress leptin secretion by a selective molecular mechanism that may contribute to the
anorexigenic effect of metformin in diabetic patients (Klein et al., 2004). So, probably the increase in adiponectin expression is coincided with an increase in PPAR-γ and HSL expression to stimulate lipolysis.

Adiponectin is well documented to reduce plasma concentration of fatty acids and triglycerides in mice models of obesity and hyperlipidemia (Yamauchi et al., 2001). The effect is mediated by acceleration of fatty acid oxidation in muscle cells, which leads to decrease in cellular triglyceride content (Fruebis et al., 2001). In skeletal muscle, adiponectin increases expression of the proteins involved in fatty acid metabolism, such as acyl-CoA oxidase and Uncoupling Protein-2 (UCP-2) and increases body temperature, suggesting a stimulatory effect on energy expenditure (Fruebis et al., 2001).

Glucocorticoids secretion from adrenal gland is primarily controlled by the hypothalamic-pituitary-adrenal axis. Glucocorticoids act in a variety of physiological processes, including the immune response, the regulation of cardiovascular activity, brain function, glucose and lipid metabolism, insulin secretion and contribute to the development of obesity and insulin resistance (Ruzzin et al., 2005). Glucocorticoids have been demonstrated to cause insulin resistance in vivo (Andrews and Walker, 1999) and are negatively regulate adiponectin expression (Halleux et al., 2001). Treatment with dexamethasone caused a decrease in adiponectin levels in serum of adrenalectomized rats, which may be related to the effects of glucocorticoids in promoting insulin resistance (de Oliveira et al., 2011b). Here, dexamethasone potentially inhibited gene expression of adiponectin and dipoR2 in adipose tissue. Therefore, adiponectin down-regulation is the mechanism by which glucocorticoids impair insulin sensitivity (Fasshauer et al., 2002) and more studies are needed to outline this alteration.

Glucose influx is inhibited in the absence of insulin and recovered on insulin treatment (Vats et al., 2004). Our findings showed that administration of insulin and metformin but not dexamethasone to type 2 diabetic rats induced a significant increase in PK and enolase mRNA expression, a key enzymes in regulation of glycolysis. The PK activity decreases due to diabetes and increases by the administration of insulin to diabetic rats in the liver tissues (Yamada and Noguchi, 1999). The increase in activity of PK in the liver tissue of rats is the cause of increased glycolysis and decreased gluconeogenesis as indicated by PEPCK mRNA expression (Taylor and Agius, 1988). Moreover, GLUT-2 expression is increased after insulin and metformin administration. GLUT-2 is trans membrane carrier protein, that enables passive transport of glucose across cell membrane. GLUT-2 regulates passage of glucose between liver and blood and it is responsible for renal glucose reabsorption (Freitas et al., 2005). Therefore, metformin up-regulate GLUT-2 expression to increase glucose metabolism in liver through up-regulation of adiponectin expression in adipose tissue.

5. CONCLUSION

In conclusion, our findings clarify that some regulators such as metformin regulated adiponectin expression to treat type 2 diabetic rats possibly at the transcriptional levels of some lipid and glucose metabolism.

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