Modulation of Skeletal Muscle Performance and Sarco-and Endoplasmic Reticulum Ca\textsuperscript{2+} ATPase by Exercise and Adiponectin Gene Therapy in Insulin Resistant Diabetic Rat Model

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ABSTRACT

This study addresses the potential application of adiponectin gene therapy and exercise in the protection against skeletal muscle dysfunction in Type 2 Diabetes Mellitus (T2DM) focusing on the role of sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) and Glut4 in pathogenesis of such dysfunction. 50 rats were divided into five groups: control, T2DM, T2DM treated with either adiponectin gene or exercise or a combination of both. Serum glucose, insulin, HOMA index, triglycerides and cholesterol were measured. Weight gain (%), muscle contractile parameters {peak twitch tension (Pt), Peak Tetanic Tension (PTT) and Half Relaxation Time (HRT)} and gene expression of SERCA, Glut4, adiponectin were assessed in gastrocnemius muscle. Results: Diabetic rats treated with either adiponectin gene or exercise showed significant reduction in serum glucose, insulin, HOMA index, triglycerides, cholesterol and weight gain. There was significant elevation in Pt and PTT with shortening in HRT. Furthermore, a significant increase in SERCA, Glut4 and adiponectin gene expression was noticed in both treated groups. Adiponectin gene therapy resulted in better muscle performance than exercise. Combination therapy caused marked gene expression of SERCA and GLUT4 and greater improvement in muscle contractility than either monotherapies. Skeletal muscle dysfunction in T2DM is mediated via impaired SERCA activity and Glut 4. Adiponectin gene therapy as well as exercise exerted partial protective effect against insulin resistance and muscle dysfunction targeting SERCA and Glut4 gene expression. Combination therapy offered the best protection against skeletal muscle dysfunction and provides a novel promising strategy for a complete cure of muscle dysfunction in T2DM.

Key words: Adiponectin, type 2 diabetes mellitus, diabetic dysfunction, adiponectin gene therapy, skeletal muscle SERCA, exercise


INTRODUCTION

T2DM is the most common metabolic disorder worldwide with a prevalence that rises markedly with increasing degrees of obesity and a sedentary life style\textsuperscript{1}. Insulin resistance is associated with a primary cellular defect in insulin action and a compensatory increase in insulin secretion\textsuperscript{2}. Adiponectin is a 244-amino acid collagen-like protein that is mainly secreted by adipocytes and acts as a hormone with anti-inflammatory and insulin sensitizing properties\textsuperscript{3}. Findings from animal studies and metabolic studies in humans suggest several mechanisms through which adiponectin may decrease the risk of T2DM. These include suppression of hepatic gluconeogenesis, stimulation of fatty acid oxidation in the liver and skeletal muscle, stimulation of glucose uptake in skeletal muscle and stimulation of insulin secretion\textsuperscript{4}. It was suggested that higher adiponectin levels are associated with a lower risk of T2DM\textsuperscript{5}.

Exercise has many well-recognized beneficial effects in healthy as well as in diabetic subjects\textsuperscript{6}. Regular exercise was recorded to improve insulin sensitivity in healthy individuals\textsuperscript{7,8}. It also improves blood glucose control and may prevent T2DM in high-risk individuals\textsuperscript{9,10}. It was shown that even with impaired insulin action, exercise-induced increase in skeletal muscle glucose uptake seemed intact\textsuperscript{11}.

Ca\textsuperscript{2+} ATPases of endoplasmic reticulum (SERCAs) are responsible for maintenance of Ca\textsuperscript{2+} ion concentrations within the endoplasmic reticulum (ER)\textsuperscript{12}. Many studies linked insulin resistance in T2DM to impaired SERCA activity. Other studies reported several common dysfunctions of Ca\textsuperscript{2+} handling and
alteration in SERCA activity in response to glucose in NIDDM model. This indicates a role of SERCA in the poor insulin secretion. Moreover, many novel links between SERCA and exercise were established. In mammalian skeletal muscle, SERCA activity has been shown to be acutely reduced after exhaustive exercise bouts. Acute exercise training also has been found to improve Ca\(^{2+}\) sequestering performance of trained-striated muscle. In addition, previous studies showed that the impairment of Sarcolemmal Reticulum (SR) function in diabetic cardiomyopathy is caused by reduced activity of SERCA2a due primarily to a decrease in SERCA2a expression.

In the present study we have employed type 2 diabetic rats with skeletal muscle weakness to determine whether adiponectin gene therapy and regular swimming exercise regimen are able to counter the progression of skeletal muscle dysfunction-related T2DM in this commonly employed animal model. We also investigated the role of SERCA and adiponectin in the pathogenesis of skeletal muscle dysfunction in this model. In addition, the links between SERCA, adiponectin to GLUT4 were studied.

MATERIALS AND METHODS

The present experiment was reviewed and approved by the Committee of Ethics of Animal Experiments and conducted according to the Guidelines for Animal Experiments, German University in Cairo, Faculty of Pharmacy and Biotechnology.

Experimental animals and protocols: Eight-week-old albino male rats were obtained from the animal house of National Research Center, Cairo, Egypt. The animals were housed in wire mesh cages at room temperature. They were fed their respective diets with free access to water. Animals were randomly divided into five groups: Control Group (CG), Diabetic Group (DG), DG treated by adiponectin gene (DAG), DG treated by swimming exercise (DEG) and DG treated by a combination of both (DAEG). T2DM was induced by administration of high fat diet (fat content 42% of energy), based on lard (HF L), olive oil (HF O), coconut fat (HF C) or fish oil (derived from cod liver, HF F) for a 12 week diet course with a single intraperitoneal injection of 45 mg kg\(^{-1}\) of streptozotocin (Sigma, St. Louis, MO.). Control rats were injected with the same volume of saline buffer. Diabetes was confirmed on the second day post-STZ administration if serum glucose concentration was at least 250 mg dL\(^{-1}\). At the end of the experimental protocol rats were sedated with an intraperitoneal injection of thiopental Sodium before surgery. The left gastrocnemius was isolated from its distal insertion, keeping nerve and vasculature intact for the assessment of the physiological muscle contraction parameters which include: Peak twitch tension, peak tetanic tension and half relaxation time.

Preparation and transfection of adiponectin gene: Adiponectin gene was taken from a muscle tissue to be amplified by RT-PCR and purified by using Qiagen Kit, where the purification solution is slightly acidic, containing guanine chloride and this enhances DNA negative charge. DNA was washed to remove small pieces of DNA. This was achieved using Ethanol and Tris buffer. This solution solubilizes smaller pieces of DNA, lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used as transfection kit and it is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dicloleolixy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipidolyl phosphatidylethanolamine (DOPE) in membrane-filtered water. Transfection activity was enhanced by using Plus™ Reagent (Cat. No 11514-015) to pre-complex the DNA. Where 0.2-0.4 µg DNA was diluted in 25 µL of Opti-MEM® I Reduced Serum medium and mixed gently. Lipofectamine™ was mixed gently before use and then 0.5-5 µL was diluted in 25 µL of Opti-MEM® I Reduced Serum medium and mixed gently. The diluted DNA was combined with diluted Lipofectamine™, mixed gently and incubated for 15-45 min at room temperature. For each transfection, 0.15 mL of Opti-MEM® I Medium was added to the tube containing the complexes and mixed gently. Finally, 0.2 mL of the diluted complexes was added by intramuscular injection.

Exercise protocol: Exercise training was carried out by long-term swim training, over a period of 9 weeks. Animal training was done in a group swimming method in half-filled cylindrical plastic container, containing water at room temperature, in session of 10 min, 5 days/week. The rats were acclimated to swimming by gradually increasing the swimming time and sessions as follows: 1st 2 weeks, 10 min, 1 session, 2nd 2 weeks, 20 min, 2 sessions, 3rd 2 weeks, 30 min, 3 sessions, last 3 weeks, 40 min, 4 sessions. Sessions were separated by 1 h rest.

Biochemical analysis: Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II (Beckman Instruments). Plasma insulin was determined using ELISA kit for the measurement of rat/mouse insulin (Linco Research, Missouri, USA). Determination of HOMA-IR: HOMA-IR was calculated as the product of fasting insulin (microunits mL\(^{-1}\)) multiplied by fasting glucose (mmol L\(^{-1}\)) divided by 22.5. Plasma triacylglycerol and cholesterol levels were measured using a triglyceride quantification kit (BioVision Research, CA, USA).
a cholesterol assay kit (BioAssay System, CA, USA). All analyses were carried out according to the manufacturers’ instructions.

Assessment of muscle physiological parameters:
Gastrocnemius muscle was isolated from its distal insertion, keeping nerve and vasculature intact and secured to a MLT0210/D force-displacement transducer by a small metal hook fastened to the calcaneal tendon. The leg was secured by screws tightened onto the medial and lateral condyles of the femur. Using a MLA0320 Stimulator, the muscle was stimulated through twin platinum electrodes that were applied directly to the surface of the muscle. Optimal voltage was determined by generating single twitch contractions at increasing voltages until no increase in single-twitch force production was observed. Optimal muscle length was determined in a similar manner. Specifically, muscle length was manipulated and single-twitch force production was observed. The length and voltage at which a single twitch produced the greatest force were used throughout the stimulation protocol. For Pt assessment, the stimulating voltage was set to produce a maximum contraction using gradual increase in the square-wave pulses from 0 to 10 V until producing maximum contraction for 1ms duration and 20 Hz frequency. Recording were displayed at sampling rate 1 K sec⁻¹. For PTT assessment, the left gastrocnemius muscles were stimulated by 15 supramaximal pulses, 1 msec duration with a gradual increase in frequency (from 20 to 50 Hz) until the muscle was completely tetanized and HRT was assessed from the maximum to half the maximum peak tension at muscle optimal length during the relaxation phase of the twitch. Muscle temperature was monitored and maintained at 35–37°C, while the muscle was kept hydrated with saline. Data were collected through an AD Instruments Bridge Amp and Powerlab 4/25 and were analyzed with Chart5 PowerLab software for Windows. The tissues were immediately collected and either snap frozen or mounted on cork using mounting medium and quick frozen in isopentane cooled by liquid nitrogen. Samples were maintained at -80°C until further analysis for detection of gene expression.

PCR analyses For RT-PCR: Thirty milligram of gastrocnemius muscle was collected at 67 days after vector infusion and snap-frozen in liquid nitrogen. Total RNA was extracted using SV Total RNA Isolation System (Promega, Madison, WI, USA) and reverse transcribed. Optimal primer and cDNA template concentrations were determined by titration. Amplification efficiencies for each primer set were determined to be similar. Data were represented as differences between threshold cycle values (ACT) for the transcripts of interest and the internal standard, α-actin. The expression of the muscle genes encoding for SERCA, GLUT4 and adiponectin were examined using semi-quantitative RT-PCR. Oligonucleotide sequences are available on request.

Statistical analyses: Differences between groups were determined by F test, ANOVA and Post hoc test. Data are expressed as Mean ± Standard Errors (SEM).

RESULTS
Effect of adiponectin gene transfer therapy and/or exercise on diabetic state: The use of high fat diet and streptozotocin resulted in an insulin resistant diabetic model (DG group) that exhibited hyperglycemia, hyperinsulinemia and increased levels of triglycerides and cholesterol in comparison to control rats. Treatment with either adiponectin gene or exercise or both resulted in a significant decrease in all these parameters compared to DG group. However, the treatment with either therapy did not improve the values of these parameters to the control values. As concerning HOMA-IR, serum triglycerides and cholesterol levels, the combination between adiponectin gene and swimming exercise resulted in a significant reduction in these parameters compared to DEG and in only serum cholesterol level when compared to DAG (Table 1).

Effect of adiponectin gene transfer therapy and/or exercise on weight gain: Induction of T2DM led to a significant increase in weight gain compared to CG. Adiponectin gene therapy or exercise resulted in a significant reduction in weight gain compared to DG but still not reaching the control values. Meanwhile, the combination therapy resulted in a significant decrease in weight gain compared to the control (Table 2).

Effect of adiponectin gene transfer therapy and/or exercise on muscle contraction parameters: Pt and PTT were significantly decreased in DG with a prolongation of HRT compared to CG. Treatment with either adiponectin gene or performing swimming exercise elevated both, Pt and PTT with shortening of HRT compared to DG. However, the present results elucidated that adiponectin gene therapy exerted a better protective role than exercise. Furthermore, the cotreatment using both therapies not only caused a significant increase in Pt and shortening of HRT compared to DG but it resulted in more significant increase in Pt and more shortening in HRT than CG. Therefore, the current data showed that the best protective effect was offered by the combination therapy (Fig. 1-3).
Table 1: Serum glucose, insulin, HOMA-IR, serum triglycerides and cholesterol levels of the five studied rat groups at the end of the experimental protocol

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CG</th>
<th>DG</th>
<th>DAG</th>
<th>DEG</th>
<th>DAEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum glucose (mm mol⁻¹)</td>
<td>3.42 ± 1.200</td>
<td>11.57 ± 1.880 *</td>
<td>7.84 ± 1.210 **</td>
<td>8.97 ± 0.927 **</td>
<td>7.38 ± 0.568 **</td>
</tr>
<tr>
<td>Serum insulin (μIU mL⁻¹)</td>
<td>11.35 ± 1.368</td>
<td>33.38 ± 6.39 *</td>
<td>22.16 ± 2.000 **</td>
<td>24.88 ± 5.029 **</td>
<td>21.43 ± 3.009 **</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.75 ± 0.778</td>
<td>17.1 ± 3.140 *</td>
<td>7.08 ± 1.128 **</td>
<td>9.64 ± 1.360 **</td>
<td>7.09 ± 1.22 **</td>
</tr>
<tr>
<td>Serum triglycerides (mg dL⁻¹)</td>
<td>67.89 ± 6.236</td>
<td>122.0 ± 2.540 *</td>
<td>27.88 ± 7.108 **</td>
<td>79.88 ± 7.160 **</td>
<td>69.23 ± 7.260 **</td>
</tr>
<tr>
<td>Serum cholesterol (mg dL⁻¹)</td>
<td>125.7 ± 5.770</td>
<td>191.6 ± 8.940 *</td>
<td>143.4 ± 5.960 **</td>
<td>159.5 ± 7.580 **</td>
<td>129.3 ± 6.250 **</td>
</tr>
</tbody>
</table>

Data are Means ± SEM, *p<0.05 vs. CG, **p<0.05 vs. DG, ***p<0.05 vs. DAG, ****p<0.05 vs. DEG.

Table 2: Weight gain at the end of the experimental protocol in the five studied rat groups

<table>
<thead>
<tr>
<th>Weight gain before sacrifice</th>
<th>CG</th>
<th>DG</th>
<th>DAG</th>
<th>DEG</th>
<th>DAEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>41.5 ± 6.392</td>
<td>59.70 ± 6.523 *</td>
<td>36.14 ± 5.633 **</td>
<td>47.72 ± 2.716 **</td>
<td>30.06 ± 5.519 **</td>
</tr>
</tbody>
</table>

Data are Means ± SEM, *p<0.05 vs. CG, **p<0.05 vs. DG, ***p<0.05 vs. DAG, ****p<0.05 vs. DEG.

Fig. 1: Peak twitch tension (Pt) in five studied rat groups

Fig. 2: Peak tetanic tension (PTT) in five studied rat groups

Effect of adiponectin gene transfer therapy and/or exercise on muscle gene expression: SERCA, GLUT4 and adiponectin muscle gene expressions were significantly lower in DG than CG. Either adiponectin gene therapy and/or exercise significantly increased expression of these genes compared to DG. Nevertheless, combination therapy proved to have better protective role concerning SERCA and GLUT4 than the use of adiponectin gene as monotherapy (Fig. 4-7).
DISCUSSION
T2DM is one of the most common metabolic disorders, representing a major health issue worldwide. Since skeletal muscles are the primary sites of insulin-dependent glucose disposal thus, resistance of the skeletal muscles to insulin-dependent glucose uptake may be an early step in the development of T2DM.

In the present study, the administration of a high fat diet and STZ injection in DG produced a significant increase in serum glucose, insulin, HOMA-IR, serum triglycerides and cholesterol levels compared to CG indicating the development of insulin resistance.

In the present study, the muscle mechanical contractile performance was assessed. Pt and PTT were recorded. Whereas Pt reflects the strength of the force and the contractile activity of the muscle, PTT signifies that impairment in force generation in fibers upon removal of external Ca²⁺ is due to impaired SR Ca²⁺ release. DG showed a significant decrease in Pt and PTT to control values. The reduction in Pt and PTT is in agreement with several studies, including that of Paulus and Grossie who recorded a marked decrease in twitch and tetanic forces in uncontrolled diabetic rats. Several mechanisms were involved to explain this deterioration. The polyol pathway was implicated and was driven by both hypoinsulinemia and hyperglycemia. Also, the hypoinsulinemia was considered as a secondary factor causing atrophy, particularly of fast muscles.

In the present study, HRT was also measured. HRT depends on the uptake of Ca²⁺ by the SR, the rate of dissociation of cross-bridges and the free energy of hydrolysis of ATP. The current results showed that HRT was prolonged in the untreated diabetic rats. Cotter reported similar results. They attributed the prolongation of HRT to the polyol accumulation which causes swelling of SR tubules and SR damage. Current results revealed a significant decrease in the gastrocnemius muscle SERCA gene expression level in

Fig. 5: GLUT4 muscle gene expression in five studied rat groups

Fig. 6: Adiponectin muscle gene expression in five studied rat groups

Fig. 7: RT-PCR analysis of transcription of the genes encoding for SERCA, GLUT4, adiponectin and γ-actin muscle gene expressions. DNA marker with 100 bp (Lane M), Lane 1: CG, Lane 2: DG, Lane 3: DAG, Lane 4: DEG, lane 5: DAEG
DG. Therefore, such alteration in SERCA gene expression and the resulting decrease in Ca^{2+} uptake by SR could explain the prolongation of HRT in DG in this study.

Depletion of GLUT4 in either adipose tissue or skeletal muscle causes insulin resistance. Therefore, in the present work, the reduction in the muscle GLUT4 gene expression in DG confirmed the insulin resistance and maybe one of the causes of the reduction in Pt and PTT as it is associated with a decrease in glucose uptake, a reduction in the energy and fuel in the muscle, resulting in the weak contraction obtained.

Treatment with adiponectin gene therapy resulted in an improvement in the diabetic state. Yet, this group values did not reach the control values suggesting partial improvement in these parameters. Another study showed similar results to our own. Adiponectin administration in both wild-type (Weight) or diabetic animal models revealed an enhancement in insulin sensitivity and corrected metabolic abnormalities. Yamauchi proved that these adiponectin effects are mediated through AMPK activation. Activation of PPAR-α, a further important factor, that regulate the expression of genes involved in glucose and lipid metabolism, was also identified to mediate the insulin sensitizing action of adiponectin.

As regards the gastrocnemius muscle contractile performance, DG treated with the adiponectin gene showed a significant increase in Pt and PTT compared to the untreated DG, with no significant difference with the control values, suggesting complete improvement in the muscle force following gene therapy. The present study is in agreement with the work of Krause, who assessed the muscle contractile properties, where PTT was 50% lower in adiponectin-KO (Ad-KO) mice compared to weight mice. Furthermore, this study revealed a shortening in HRT in DG on gene therapy, suggesting an increase in Ca^{2+} uptake by the SR.

In addition, the present study proved an increase in the muscle SERCA, GLUT4 and adiponectin gene expressions in the treated DG with the adiponectin gene. Thus, the shortening of HRT can be explained by the significant increase in the SERCA gene expression and consequently the increase in the Ca^{2+} uptake by SR. It has been postulated that adiponectin may attribute to the enhanced glucose uptake in skeletal muscle cells via increasing GLUT4 expression and translocation.

In the present study, we examined the effects of a 9 weeks daily swimming exercise regimen on T2DM rats. DG following the swimming exercise regimen showed a significant reduction in the measured serum parameters compared to DG. Yet, this same group showed a significant elevation in all these measured parameters compared to CG, suggesting a partial protection.

Pt and PTT showed a significant increase in the trained DG compared to the untrained DG and a significant decrease compared to CG. Moreover, this same group showed a shortening in HRT compared to DG.

Moreover, the trained DG revealed a significant increase in the muscle SERCA, GLUT4 and adiponectin gene expressions. This coincides with other studies which indicated that both moderate and high intensity exercise significantly increase the SERCA2a expression in the gastrocnemius. Exercise training has profound effects on PPAR-γ activation in rats. Activation of PPAR-γ affects GLUT4, activates AMPK and improves insulin action. Since PPAR-γ activation is also associated with increased adiponectin production, activation of PPAR-γ with exercise represent a possible mechanism that links the insulin sensitizing effects of exercise with adiponectin.

Furthermore, the combination of adiponectin gene therapy and exercise resulted in a significant reduction in all the serum parameters.

The present study also revealed a significant elevation in Pt and PTT with a shortening in HRT in DG on the combined therapies compared to both DG and CG, suggesting full improvement in the muscle contractile performance by the combination therapy.

Finally, the combination therapy group showed a significant increase in the gastrocnemius muscle SERCA, GLUT4 and adiponectin gene expressions. An interesting observation in the previous studies was that both exercise and adiponectin shared somewhat similar insulin sensitizing effects. Both promoted skeletal muscle glucose uptake, increased fatty acid oxidation and modulated GLUT4 translocation and AMPK activity.

CONCLUSIONS

To present study, this is the first study linking SERCA and skeletal muscle contractile performance in a T2DM insulin resistant animal model. There was alteration in SERCA, GLUT4 and adiponectin genes expression in the gastrocnemius muscle associated with contractile dysfunction. Adiponectin gene therapy or swimming exercise, by increasing the expression of these genes, exerted a partial protective effect on the insulin resistance state and the skeletal muscle contractile performance. However, the use of a combination therapy resulted in the best protection against the skeletal muscle deterioration. Taken together these data support the potential use of such a combination as a promising strategy in the treatment of T2DM muscle dysfunction.

RECOMMENDATIONS

Further investigations on the role of adiponectin gene therapy and exercise training in other
pathophysiologic pathways involved in diabetic skeletal muscle dysfunction are recommended. In addition, the trial of various types of exercise protocols is recommended in order to select the one which provides the best protection available.

**NOMENCLATURE**

HOMA-I = Homeostasis model assessment of insulin resistance
HRT = Half relaxation time
Pt = Peak twitch tension
PTT = Peak tetanic tension
SERCA = Sarco and endoplasmic reticulum Ca$^{2+}$ ATPase
T2DM = Type 2 diabetes mellitus

**REFERENCES**


