Changes in blood biochemical parameters and in gene expression in skeletal muscle after therapeutic exercise in diabetic dogs

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Abstract: Therapeutic exercise is a beneficial treatment for diabetes mellitus that ameliorates insulin sensitivity and increases glucose uptake into skeletal muscle. In this study, we investigated whether therapeutic exercise affects blood biochemical parameters and muscular mRNA expression in diabetic dogs. No significant difference was observed between the fasting blood glucose concentrations before and after exercise. However, the levels of glycate albumin (GA) and non-esterified fatty acids were significantly decreased after exercise. No significant differences were found between before and after exercise in the mRNA expression of insulin signaling and glucose metabolism genes including insulin receptor substrate (IRS)-1, IRS-2, phosphatidylinositol 3-kinase, Akt kinase 2, glucose transporter 4, AMP-activated protein kinase, uncoupling protein 3, and acetyl-CoA carboxylase. In summary, therapeutic exercise decreased GA levels and thus ameliorated glycemic control in diabetic dogs.


Key word: diabetes mellitus, dog, glycemic control, therapeutic exercise

Introduction

Insulin and exercise are the two most physiologically relevant stimulators of skeletal muscle glucose transport [5,6,13]. Both insulin and exercise, or muscle contraction, increase glucose uptake by skeletal muscle. Insulin signaling involves the rapid phosphorylation of the insulin receptor and of insulin receptor substrate (IRS) 1 and 2 on tyrosine residues and the activation of phosphatidylinositol 3-kinase (PI3-K) [2,4]. In contrast, exercise and muscle contraction have no effect on insulin receptor or IRS-1 phosphorylation or on PI3-K activity[19]. The AMP-activated protein kinase (AMPK) is a protein implicated in muscle glucose transport in response to changes in the cellular energy status. AMPK has been implicated in the regulation of muscle fiber type[3,12,17], mitochondrial biogenesis[23], and GLUT4 biogenesis in skeletal muscle [7], making AMPK a key protein of interest in the study of exercise-mediated muscle adaptations. Therefore, exercise increases the expression of GLUT4 at the mRNA and protein levels.

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The therapeutic benefits of exercise in human diabetes mellitus (DM) patients are well known. Therapeutic exercise in human DM patients is associated with improved insulin action (increased insulin sensitivity) in skeletal muscle, lowered blood glucose levels, reduced hemoglobin A1c (HbA1c) levels, and reduced body fat [26]. Additionally, exercise activates AMPK, which phosphorylates and thereby inhibits acetyl-CoA carboxylase, resulting in reduced malonyl-CoA content and hence enhanced fatty acid oxidation. There has been much research on the relationship between therapeutic exercise and glycemic control in humans. However, the relationship is not clear in dogs.

The purpose of this study is to evaluate whether therapeutic exercise has effects on blood biochemical parameters and muscular mRNA expression in dogs with DM.

**Materials and Methods**

**Animals**

Three dogs with DM maintained in our laboratory were used in this study (one female miniature schnauzer aged 4 years, one castrated male miniature dachshund aged 6 years, and one male beagle aged 8 years). All dogs were fed a commercial diet (Select Protein; Royal Canin Japon, Inc., Tokyo, Japan) twice a day (at 8 am and 8 pm), and the caloric intake was set at half of $2.0 \times \text{RER (BW^{0.75} \times 70)}$ for each feeding period, where RER is the resting energy requirement and BW is the body weight of the diabetic dog. The dogs were injected postprandially with different amounts of neutral protamine Hagedorn insulin (NPH insulin Novolin N; Novo Nordisk Pharma Ltd., Tokyo, Japan) in the range of 0.48–0.69 IU/kg according to their glycemic control (Table 1). The same amounts of insulin were administered both before and after the 4-week exercise program. Approval for this work was given by the Nippon Veterinary and Life Science University Animal Research Committee.

**Exercise**

The exercise program consisted of walking (4 km/h, 18 min) and running (6 km/h, 2 min) at room temperature and constant humidity. This exercise training was performed between 5:00 pm and 5:20 pm 6 days a week (Monday through Saturday) for 4 weeks.

**Blood sampling and assaying**

Blood samples were obtained from the jugular vein of each dog at 0 (preprandially), 2, 4, 6, 8, 10, and 12 h after feeding and insulin injection on the day before the start of the exercise program (0 week) and after 1, 2, 3, and 4 weeks of exercise on Saturdays. Blood samples were collected into heparinized plastic tubes or polypropylene tubes. These samples were centrifuged at 1,700 g for 10 min at 4°C to obtain plasma and serum, which were immediately stored at −80°C until further use. Plasma samples were used to measure glucose and adiponectin levels. Serum samples were used to measure the levels of glycated albumin (GA), 3-hydroxybutyric acid (3-HB), and non-esterified fatty acids (NEFA). Plasma glucose concentrations were measured using the Glucose Test Wako2 kit (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Plasma adiponectin concentrations were measured using the Mouse/Rat Adiponectin ELISA kit (Otsuka Co., Ltd., Tokyo, Japan). Serum GA, 3-HB, and NEFA were measured using commercial kits—Lucica GA-L (Asahi Kasei Pharma Corp., Tokyo, Japan) and Autosera ALB-1 (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan), N-Assay 3-hydroxybutyric acid (Nittobo Medical Co., Ltd., Fukushima, Japan), and IATROTEC NEFA (Mitsubishi Chemical Medience Corp., Ltd., Tokyo, Japan), respectively—and processed by a Type 7180 Automatic Analyzer (Hitachi High-Technologies Corp., Ltd., Tokyo, Japan).

**Table 1 Diabetic dogs used in this study**

<table>
<thead>
<tr>
<th>Breed</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Body Weight (kg)</th>
<th>NPH Insulin (IU/kg/BID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.1 M. schnauzer</td>
<td>4</td>
<td>Female</td>
<td>6.25</td>
<td>0.48</td>
</tr>
<tr>
<td>No.2 M. dachshund</td>
<td>6</td>
<td>Neutered Male</td>
<td>5.92</td>
<td>0.68</td>
</tr>
<tr>
<td>No.3 Beagle</td>
<td>8</td>
<td>Neutered Male</td>
<td>11.6</td>
<td>0.69</td>
</tr>
</tbody>
</table>
**Collection of tissue samples**

Dogs were sedated by intravenous administration of 0.25 mg/kg droperidol (Droletan; Daiichi Sankyo Co., Ltd., Tokyo, Japan) and anesthetized by intravenous administration of 7 mg/kg propofol (Rapinovet; Intervet/Schering-Plough Animal Health Corp., Ltd., Tokyo, Japan). Anesthesia was maintained by inhalation of isoflurane (Esccain; Merck Ltd., Tokyo, Japan) and oxygen.

Skeletal muscle samples were collected 2 weeks before and after 4 weeks of exercise training. Two hundred milligrams of biceps femoris muscle was removed by biopsy from each anesthetized animal under minimal-stress conditions according to the guidelines of the Nippon Veterinary and Life Science University. The tissue sample was immediately transferred into RNAlater solution (Sigma, St. Louis, MO, USA) and stored at −80°C until further use.

**Quantitative real-time PCR analysis of mRNA**

Quantitative real-time PCR (qRT-PCR) analysis was used for comparing mRNA levels in skeletal muscle before and after exercise. Total RNA was extracted by homogenizing skeletal muscle in TRIzol reagent (Invitrogen, Tokyo, Japan). The primers used in qRT-PCR were designed from GenBank sequences (Table 2).

The qRT-PCR reactions for each gene of interest were performed in triplicate with β-actin serving as an internal standard. Reactions were carried out with Perfect Real Time SYBR Premix Ex Taq (Takara, Siga, Japan) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the conditions of 94°C for 10 s followed by 35 cycles of 95°C for 5 s and 60°C for 34 s. Each 20-µL PCR reaction contained 2 µL of template cDNA, 0.4 µL of each specific primer, 10 µL of SYBR Premix Ex Taq, 0.4 µL of ROX reference dye, and 6.8 µL of distilled water. After qRT-PCR amplification, absolute quantification was performed according to the method of Whelan et al.[22], by establishing a linear amplification curve from tenfold serial dilutions of cloned and sequenced plasmid DNA. Each value of mRNA expression was calculated and expressed as copies.

**Statistical analysis**

Data are presented as mean±standard deviation (SD). Total area under the curve (AUC) for glucose was calculated by the trapezoidal rule. Statistical significance
was determined by a paired Student’s t test, one-way repeated-measures ANOVA, Dunnett’s multiple comparison test, or two-way repeated-measures ANOVA using GraphPad Prism 5 analysis software (GraphPad Software, San Diego, CA, USA). The significance level was set at p < 0.05.

**Results and Discussion**

Total area under the curve during 0–12 h (glucose AUC_{0-12h}) was estimated as the postprandial summary variable. Fasting blood glucose (FBG) concentration, glucose AUC_{0-12h}, GA, and 3-HB level were evaluated at 0, 1, 2, 3, and 4 weeks of therapeutic exercise. Temporal analysis of postprandial glucose concentration at 0 week and after therapeutic exercise showed no difference in glucose concentration over time (Fig. 1-a, two-way repeated-measures ANOVA). Moreover, no difference was observed in mean (±SD) FBG and glucose AUC_{0-12h} (Fig. 1-b and 1-c, one-way repeated-measures ANOVA). However, serum GA levels were significantly decreased after therapeutic exercise in diabetic dogs (Fig. 1-d, p < 0.05, one-way repeated-measures ANOVA). Moreover, a significant difference (p < 0.05, Dunnett’s multiple comparison test) was observed at 3 and 4 weeks relative to 0 week.

FBG levels and glucose AUC_{0-12h} profiles were not significantly changed from 0 week to various weeks after therapeutic exercise. FBG and glucose AUC_{0-12h} indicate temporal glycemic status (at just one point in time or on one day), whereas GA is the primary glycemic control marker in veterinary medicine. Serum GA level can provide an index of glycemic control for 1–3 weeks in dogs [14,15]. As such, a decreased GA level after therapeutic exercise is indicative of ameliorated glycemic control. Therefore, we demonstrated that glycemic control improved from therapeutic exercise on the basis of GA levels. Therapeutic exercise is reported to decrease FBG and HbA_{1c} levels in patients with type 1 DM. The duration of exercise programs in human studies, which is generally over 6 months, is longer than that in our study [16,20].

Our present study involving 4 weeks of exercise training for 6 days per week demonstrated that serum GA levels significantly decreased after therapeutic exercise, but FBG levels had no significant changes. Therefore, we think that long-term exercise might be necessary to reduce FBG levels. Moreover, it is also known that although exercise can reduce blood glucose levels in patients with type 1 DM, it can also lead to hyperglycemia and ketosis under various exercise conditions, depending on insulin availability [1,21]. Additionally, it has been reported that the timing, term, and time of exercise are important for

![Fig. 1](image-url) Changes in blood biochemical parameters in three dogs with diabetes mellitus after exercise training. Mean temporal plasma glucose concentration (a), fasting blood glucose concentration (FBG) (b), total area under the curve for glucose during 0–12 h (glucose AUC_{0-12h}) (c), glycated albumin (GA) (d), 3-hydroxybutyric acid (3-HB) (e), and non-esterified fatty acids (NEFA) (f) were determined before (0 week) and after exercise training (1, 2, 3, and 4 weeks). Plasma adiponectin concentration (g) was also determined before (0 week) and after exercise (4 weeks). All results are expressed as mean±standard deviation (SD). An asterisk indicates a significant difference (p < 0.05, paired t test) relative to the control value (0 week).
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glycemic control in patients with type 1 DM[9]. In this study, a significant difference was not observed in blood glucose or ketone body (3-HB) concentration under therapeutic exercise (Fig. 1-e). This result might indicate that our therapeutic exercise protocol was too mild for the diabetic dogs or that the dogs had moderate exercise tolerance.

Serum NEFA concentrations were significantly decreased after exercise training (Fig. 14, p<0.05, one-way repeated-measure ANOVA). Fatty acids are an important oxidative fuel both at rest and during exercise [8]. Exercise is a very potent lipolytic stimulus because of the need to meet the increased energy requirements due to muscle work. During exercise, the whole-body lipolytic rate and blood NEFA availability increase approximately 5-fold[11,24]. Thus, an increase in NEFA availability from therapeutic exercise might be related to the decrease in serum NEFA concentration in our study. Furthermore, NEFA are known to impair insulin-stimulated glucose uptake; therefore, our result might indicate an improvement in insulin sensitivity in the diabetic dogs.

Plasma adiponectin concentrations did not change significantly before and after exercise (Fig. 1-g). Adiponectin is a protein synthesized and secreted by adipocytes. A decreased serum adiponectin level is responsible for insulin resistance associated with obesity. Recent studies have shown that adiponectin improves insulin sensitivity through activation of the AMP kinase pathway[10,25]. Therefore, the relationship between adiponectin level and intensity and duration of therapeutic exercise should be further studied under different conditions.

The gene expression levels of IRS-1, IRS-2, PI3-K, AKT2, AMPK, GLUT4, ACC, and UCP3 did not change significantly with therapeutic exercise (Fig. 2 a–h). Thus, the present study did not find significant changes in the expression of insulin signaling and glucose and lipid metabolism genes after exercise. These results are consistent with that reported in a past human study on the expression of insulin signaling genes, but are inconsistent with that found on the expression of glucose and lipid metabolism genes. AMPK activity has been observed to be higher in high-intensity exercise than in low-intensity exercise in mice. In the case of low-intensity exercise, the low AMPK activity necessitates exercising for a long time to have enough momentum to increase GLUT4 contents [18]. The exercise program in the present study might be comparatively low intensity and short term, because it did not bring about significant changes in AMPK and GLUT4 gene expression. Changes in the term, intensity, and time of exercise will help elucidate the relationship between gene expression and exercise. Another limitation of our study is a low statistical power attributed to the small number of diabetic animals used in this study. Because of the large biological variability among animals and the small

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**Fig. 2** Changes in the mRNA levels of glucose and insulin metabolism signaling genes in the skeletal muscle of diabetic dogs. mRNA levels were determined by quantitative real-time-PCR for insulin receptor substrate (IRS)-1 (a), IRS-2 (b), phosphatidylinositol 3-kinase (PI3-K) p85α (c), Akt kinase 2 (AKT2) (d), acetyl-CoA carboxylase (ACC) (e), uncoupling protein 3 (UCP3) (f), glucose transporter 4 (GLUT4) (g), and AMP-activated protein kinase (AMPK) (h). Results are expressed as mean±standard deviation (SD). Muscle samples collected both before exercise (pre) and after exercise (post) were analyzed.
sample size, it is not possible for this study to accurately assess the true efficiency of therapeutic exercise.

In conclusion, therapeutic exercise decreased the GA level and thus improved glycemic control in diabetic dogs. However, the relationship between therapeutic exercise and muscular gene expression should be further studied.

References


糖尿病犬における運動療法が血液生化学パラメターおよび骨格筋遺伝子発現の変動に与える影響

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要 約：運動療法は、インスリン感受性を改善し、骨格筋へのブドウ糖取り込みを増加させることから、糖尿病患者において有用な治療法である。本研究では、糖尿病犬に対する運動療法が血液生化学パラメーターおよび骨格筋遺伝子発現にどのような影響を及ぼすかを検討した。空腹時血糖値は運動前後で有意な変動は認められなかった。しかし、糖化アルブミン（GA）および遊離脂肪酸は運動後有意に低下した。インスリンシグナルがおおよび糖代謝に関連する遺伝子（インスリンレセプター基質1および2、ホスファチジルイソシトール3キナーゼ、aktキナーゼ2、グルコーストランスポーター4、AMP活性化プロテインキナーゼ、脱共役化蛋白3およびアセチルCoAカルボキシラーゼ）の発現には運動前後で有意な変化は認められなかった。本実験結果より、運動療法を行うことでGAの低下をもたらし、糖尿病犬において血糖コントロールが改善された。


キーワード：diabetes mellitus, dog, glycemic control, therapeutic exercise

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