Effect of testosterone and endurance training on glycogen metabolism in skeletal muscle of chronic hyperglycaemic female rats

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Original Article

Objective: To investigate in glycolytic and oxidative muscles of trained (nine weeks) and untrained hyperglycaemic female rats the effect of hyperandrogenicity and/or endurance training on energy metabolic properties.

Methods: Glycogen content and activity of muscle enzymes with regulatory functions in glycogen synthesis were examined.

Results: Testosterone treatment increased glycogen content of extensor digitorum longus (EDL) and soleus muscles of hyperglycaemic sedentary (18% and 84% respectively) and hyperglycaemic trained (7% and 16% respectively) rats. In both types of muscle of the hyperglycaemic testosterone treated exercised subgroup, less depletion of glycogen was found than in the untreated group (38% and 87% for EDL and soleus respectively).

Conclusions: The mechanisms by which training and/or hyperandrogenism alone or in combination elicits their specific effects are complex. Differences in sex, surgery, levels of hormones administered, and exercise model used may be the main reasons for the observed discrepancies. Conclusions from the results: (a) hyperandrogenism is not a primary cause of the development of insulin resistance; (b) hyperglycaemic control of slow and fast twitch muscle is increased by training through increased glycogen synthase activity. The most plausible explanation for differences between different muscle fibre types is the different levels of expression of androgen receptors in these fibres. Hyperandrogenicity therefore acts on energy metabolic variables of hyperglycaemic animals by different mechanisms in glycolytic and oxidative muscle fibres.

Type 2 diabetes mellitus is a disorder characterised by miscellaneous humoral and/or metabolic derangements such as impaired pancreatic insulin secretion and impaired peripheral tissue sensitivity to insulin (insulin resistance). For instance, in clinically overt type 2 diabetes mellitus, muscle resistance to insulin tends to cause decreased rates of glucose uptake, leading to hyperglycaemia. Under normal conditions, hyperglycaemia has been shown to increase nonoxidative glucose disposal, by virtue of its mass-action effect, through activation of the key rate limiting enzyme of glycogen synthesis, glycogen synthase. Long term persistent hyperglycaemia may eventually lead to insulin resistance and reduced glycogen synthase activity. In addition, suppression of non-oxidative glucose metabolism (glycogenesis), as observed in patients with type 2 diabetes mellitus and hypertriglyceridaemia, results from increased fatty acid metabolism. On the basis of findings in epidemiological human and animal studies, hyperglycaemia and hyperandrogenism have also been suggested to be related to the insulin resistant state. For instance, sex hormones may play a prominent role in the development of insulin resistance and hence type 2 diabetes mellitus. Particularly in women with type 2 diabetes mellitus and hyperglycaemic women with polycystic ovaries and/or acanthosis nigricans, hyperandrogenism has been suggested to play an important role in the development of the pathological condition. However, these findings could not be confirmed in studies on intact, testosterone treated female rats. These investigations showed that testosterone potentiated rather than decreased the effect of training on the glycogen content of oxidative (soleus) and glycolytic (extensor digitorum longus (EDL)) muscles. Furthermore, it has been found that testosterone reduces glycogen breakdown during exercise. In contrast with earlier studies, the latter findings do not indicate a role for testosterone in the development of insulin resistance. As the above studies were performed in non-hyperglycaemic animals, the question was raised whether testosterone could elicit similar effects in streptozotocin induced, mildly hyperglycaemic, trained rats. Although the streptozotocin model may have mechanistic problems and does not always lead to lower glycogen levels, most studies have used this model to induce a level of hyperglycaemia that resembles type 1 rather than type 2 diabetes mellitus. Therefore, the aim of this study was to investigate the effects of hyperandrogenicity on energy metabolic variables of glycolytic (EDL) and oxidative (soleus) skeletal muscles of streptozotocin induced, mildly hyperglycaemic female rats. A further aim was to establish the patterns of glycogen metabolism during training and acute exercise in the above muscles.

Materials and Methods
Animal treatment and experimental procedures
The study was approved by the local ethics committee according to national and international guidelines and laws for research on animals.

Abbreviations: EDL, extensor digitorum longus; NS, non-hyperglycaemic sedentary control; NT, non-hyperglycaemic testosterone treated; HS, hyperglycaemic sedentary control; HT, hyperglycaemic testosterone treated; HTc, hyperglycaemic trained control; HTt, hyperglycaemic trained testosterone treated; HTtExC, hyperglycaemic exercised control; HTtExT, hyperglycaemic exercised testosterone treated; GSi, active portion of glycogen synthase; GSi+d, total glycogen synthase; GPa, active form of glycogen phosphorylase; GPa+a, total phosphorylase activity
A total of 72 female Brown-Norway rats were obtained immediately after birth. According to the streptozotocin hyperglycaemic rat model of Portha et al., pups were injected intraperitoneally with streptozotocin (100 mg/kg; Boehringer, Mannheim, Germany) in 50 µl sodium citrate buffer (pH 4.5) within 24 hours of birth. Control litters received sodium citrate buffer alone. At the age of 4 weeks, control and streptozotocin induced pups were weaned and housed in groups of four in a climate controlled room (mean (SD) temperature 22°C, humidity 50–60%) on a 12 hour dark/12 hour light cycle. A standard laboratory chow (54.2% carbohydrate, 7.3% fat, and 27.5% protein) and water were provided ad libitum. Rats with a fasting blood glucose concentration > 9 mmol/l were included in the hyperglycaemic group. Validation of this protocol was assessed by confirmation of the hyperglycaemic state in a subgroup of rats by an oral glucose tolerance test (1 g/kg body weight). The data from this test showed that our state in a subgroup of rats by an oral glucose tolerance test (1 g/kg body weight) was expressed as mmol/l. The controls were sham treated with an empty implant.

To study the training induced effects of our interventions, the rats were anaesthetised with light ether three days after the last training bout (HTEC and HTT), whereas to study the acute effects the rats were immediately anaesthetised after the last submaximal training session. The soleus muscle, consisting mainly of slow twitch type 1 fibres (78 (5)%), and the EDL muscle, consisting mainly of fast twitch type 2 fibres (97 (1)%), were dissected free from the right or left hindlimb, which took about two minutes. Next, 2 ml blood was withdrawn from the animal. The animal was then killed by cervical dislocation. To avoid differences caused by diurnal variation, rats from the other (sub)groups were killed on different days at the same time (0900–1000). One set of hindlimb muscles from each animal was immediately frozen in liquid nitrogen and used for the determination of glycogen, and the muscles from the contralateral hindlimb were freshly homogenised (50 mg/ml) in 250 mM sucrose/2 mM EDTA/10 mM Tris/HCl buffer, pH 7.4. The homogenate was sonicated and stored at −80°C until further analysis.

Analytical methods

Plasma testosterone concentration was determined in duplicate using a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, California, USA). The intra-assay coefficient of variation, calculated from the replicates, was 7.5% at 5 nmol/l and 7.1% at 8 nmol/l. Insulin concentrations were assessed in duplicate using a commercially available radioimmunoassay kit containing a rat insulin standard (Diagnostic Products Corporation). The intra-assay coefficient of variation, calculated from the replicates, was 3.5% at 55.4 µU/ml. The interassay coefficient of variation was 8%.

Plasma glucose (Boehringer Mannheim; 124010), plasma fatty acids (Wako Chemicals GmbH, Neus, Germany), plasma glycerol (Boehringer Mannheim), and plasma triacylglycerols (Boehringer Mannheim) were all assayed spectrophotometrically on a centrifugal analyser (Cobas Fara; Hoffman-La Roche, Basel, Switzerland) using commercially available kits. Concentrations were expressed as mmol/l (glucose, fatty acids, glycerol) and µmol/l (triacylglycerols).

Glycogen content was assayed in the freeze dried soleus and EDL muscles as described by Passonneau and Luderdale and was expressed as µmol glucosyl units/g dry weight.

The active portion of glycogen synthase (GSI) was assayed in the muscle homogenate at 30°C in the absence of glucose.
6-phosphate, and total glycogen synthase (GSi+d) by including 10 mmol/l glucose 6-phosphate. The reaction was started by the addition of UDP-glucose, and formation of UDP was measured enzymically by the method of Danforth.\textsuperscript{21} The active form of glycogen phosphorylase (GPa+b) was assayed at 30°C in the absence of AMP and total phosphorylase activity (GPa+b) by including 5 mmol/l AMP. The formation of hexose phosphates was measured spectrophotometrically as described by Harris et al.\textsuperscript{22} Both methods have been described in detail previously,\textsuperscript{23} with activities expressed as μmol/min per g wet weight.

**Statistical analysis**
Results are expressed as mean (SD) for the indicated number of animals. Data were analysed using a two way analysis of variance. The Newman-Keuls post hoc test was used to locate significant differences among the groups. The level of significance was set at \( p<0.05 \).

**RESULTS**
Table 1 shows the baseline endocrine and metabolic characteristics of control and streptozotocin induced hyperglycaemic rats at the end of the experimental period. The hyperglycaemic rats had a significantly lower body weight, a higher fasting plasma glucose concentration in combination with a significantly lower body weight, a lower fasting plasma insulin concentration and a lower fasting plasma insulin to glucose ratio.

Furthermore, the hyperglycaemic animals showed a depressed (by about 22%) insulin response to an oral dose of glucose at 15 and 30 minutes (data not shown), the sedentary hyperglycaemic rats showed increased plasma concentrations of fatty acids and glycerol. Interestingly, plasma concentrations of glucose, insulin, fatty acids, glycerol, and triacylglycerols in the trained and/or testosterone treated hyperglycaemic animals were not different from their corresponding control groups (data not shown).

Plasma testosterone levels in the untreated rats were the same in the non-hyperglycaemic and hyperglycaemic animals (0.9 (0.2), 0.9 (0.2), 1.0 (0.1), and 1.1 (0.2) nmol/l, for NS, HS, HTrC, and HTrExC groups respectively). In the testosterone treated animals, plasma testosterone levels were significantly higher (5.7 (1.6), 4.9 (1.3), 4.8 (1.5), and 5.1 (1.1) nmol/l for NT, HT, HTrE, and HTrExT groups respectively; \( p<0.005 \)).

During the final three weeks of the nine week training period, all trained rats were capable of running continuously for 60 minutes at a pace of 30 m/min (cf the work of van Breda et al\textsuperscript{19}).

**Figure 2** Glycogen content in (A) the extensor digitorum longus muscle and (B) the soleus muscle in the experimental groups. Data represent the mean (SD). NS, Non-hyperglycaemic sedentary control (\( n=12 \)); NT, non-hyperglycaemic sedentary plus testosterone (\( n=8 \)); HS, hyperglycaemic sedentary control (\( n=10 \)); HT, hyperglycaemic sedentary plus testosterone (\( n=8 \)); HTrC, hyperglycaemic trained control (\( n=10 \)); HTrT, hyperglycaemic trained plus testosterone (\( n=9 \)); HTrExC, hyperglycaemic trained exercised (60 minutes) control (\( n=9 \)); HTrExT, hyperglycaemic trained exercised (60 minutes) plus testosterone (\( n=6 \)), *\( p<0.05 \).
only in the soleus was a significantly higher glycogen content (50%) observed than in its control. In both muscles the exercise induced reduction in glycogen content was significantly less in the testosterone treated group (15% and 76% for EDL and soleus muscle respectively) than in the untreated group (38% and 87% for EDL and soleus muscle respectively).

Response of muscle glycogen synthase and glycogen phosphorylase activities to diabetes, training, and testosterone treatment

Tables 2 and 3 show the activities of glycogen synthase and glycogen phosphorylase in EDL and soleus muscles respectively. The lower glycogen content of these muscles in sedentary induced reduction in glycogen content was significantly less in the testosterone treated group (15% and 76% for EDL and soleus muscle respectively) than in the untreated group (38% and 87% for EDL and soleus muscle respectively).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Glycogen synthase and glycogen phosphorylase activity in extensor digitorum longus muscle</th>
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<tr>
<td></td>
<td><strong>Glycogen synthase</strong></td>
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<td></td>
<td><strong>Group</strong></td>
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<tr>
<td>Sedentary</td>
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<tr>
<td>Testosterone</td>
<td>2.38 (0.3)</td>
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<tr>
<td>Hyperglycaemic</td>
<td>0.79 (0.1)</td>
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<td>Hyperglycaemic + testosterone</td>
<td>1.58 (0.2)</td>
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<tr>
<td>Hyperglycaemic + trained</td>
<td>1.38 (0.1)</td>
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<tr>
<td>Hyperglycaemic + trained + testosterone</td>
<td>1.69 (0.2)</td>
</tr>
<tr>
<td>Hyperglycaemic + exercised</td>
<td>1.50 (0.2)</td>
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<tr>
<td>Hyperglycaemic + exercised + testosterone</td>
<td>2.28 (0.2)</td>
</tr>
</tbody>
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Values are mean (SD) and are expressed as µmol/min per g wet weight and as % of the total activity. *p<0.05 sedentary v hyperglycaemic; †p<0.05 hyperglycaemic v hyperglycaemic + testosterone; ‡p<0.05 hyperglycaemic v hyperglycaemic + trained; §p<0.05 hyperglycaemic + exercised v hyperglycaemic + exercised + testosterone.

DISCUSSION

This study was designed to investigate whether administration of testosterone affects the energy metabolic properties of skeletal muscle of hyperglycaemic (trained) female rats. In our model, rats were made hyperglycaemic by injection of streptozotocin within 24 hours of birth. After 8–10 weeks, the animals showed hypoinsulinaemia, hyperglycaemia, and peripheral insulin resistance, which is in agreement with previous observations.12 Although the streptozotocin model has been questioned with respect to its diabetic characteristics,12 our study clearly shows that a streptozotocin injection in pups leads to mild hyperglycaemia, which is often the first complication in the development of type 2 diabetes mellitus.

Effects of hyperglycaemia on muscle glycogen content and metabolism

Our results showing decreased muscle glycogen content and glycogen synthase activities in the hyperglycaemic rats...
support previous findings.\textsuperscript{15–20} In contrast with previous studies,\textsuperscript{4, 25–28} our findings in rats, as well as recent findings in humans,\textsuperscript{10} indicate that the mass-action effect of hyperglycaemia is not sufficient to compensate for the intracellular defects in glycogen synthesis during hyperglycaemia. Furthermore, our data show that, in the hypoinsulinaemic state, caused by chronic hyperglycaemia, the increased percentage of GSI is insufficient to compensate for the metabolic defects in type 2 diabetes mellitus. As in our model the animals were hypoinsulinaemic, it is tempting to speculate that the mass-action effect of hyperglycaemia on muscle metabolism only occurs in the presence of, at least, basal insulin levels. Recently, Bruce et al\textsuperscript{39} reported that increased glucose availability in insulin resistant Zucker rats after glycaemic depleting exercise resulted in glycogen supercompensation. However, in contrast with the model used by Bruce et al, the animals in our model were hyperglycaemic but probably not insulin resistant.

Effects of training and testosterone treatment on muscle glycogen content and metabolism

We have previously shown that the glycogen content of EDL muscle, but not soleus muscle, in non-hyperglycaemic trained female rats treated with pharmacological doses of testosterone was increased, in concert with an increase in GSI, compared with untreated rats.\textsuperscript{38} In contrast, the present study shows a higher glycogen content in both muscles in concert with a significant increase in GSI+Δ, but only in the soleus muscle in concert with a significant increase in GPa. We cannot explain the increased glycogen content in the EDL without a concomitant increase in GSI activity, but it should be remembered that the animals were killed three days after the last exercise bout. Thus, in the hyperglycaemic animals, the glycogen content may have already returned to its baseline level and likewise GSI activity. Support for this hypothesis is provided by the results of Allenberg et al,\textsuperscript{22} who reported a large effect of muscle glycogen stores on the percentage of GSI.

Glycogen sparing effect of testosterone during submaximal exercise and the mechanisms responsible

Another striking finding of our study is the glycogen sparing effect of testosterone in EDL and soleus muscles after a submaximal exercise bout in concert with an increase in GSI activity and a reduction in GPa activity. This is in line with our findings in non-hyperglycaemic animals.\textsuperscript{10} The mechanism by which testosterone regulates glycogen metabolism during exercise in hyperglycaemic rats remains obscure. The most plausible explanation is substrate competition, by which increased availability of one substrate restrains consumption of the other. This mechanism, originally demonstrated by Randle et al,\textsuperscript{12} has been shown to operate in skeletal muscle.\textsuperscript{37} As androgens have been shown to increase lipolytic activity,\textsuperscript{30} it can be argued that the glycogen sparing effect of testosterone is caused by increased fatty acid supply and subsequent oxidation. Further support for the preferential use of fatty acids over glucose can be derived from the study of Kendrick et al,\textsuperscript{35} who observed higher plasma fatty acid concentrations after exercise in non-hyperglycaemic rats treated with oestradiol-17β than in control rats. In our study, however, no differences in plasma fatty acid concentration were observed in the testosterone treated exercised streptozotocin induced hyperglycaemic rats and the untreated animals. This may be because of the lower availability of plasma fatty acids during exercise in the trained compared with the untrained state after 90 and 120 minutes of exercise.\textsuperscript{27}

Differences between glycolytic and oxidative muscle fibres

An important finding of this study is that testosterone elicits quantitatively different effects on energy metabolic properties in glycolytic (EDL) and oxidative (soleus) muscle fibres. These effects seem to result from different mechanisms in the different muscles. In the EDL muscle the sparing of muscle glycogen is caused by increased activity of GSI in concert with a small increase in GPa activity, whereas in soleus muscle decreased activity of GSI in concert with a larger decrease in GPa activity probably results in the glycogen sparing effect of testosterone during exercise.

Mechanisms and concluding remarks

Our findings also show that mild hyperandrogenicity acts on muscle metabolism in hyperglycaemic-hypoinsulinaemic streptozotocin-hyperglycaemic female rats. Particularly in the trained hyperglycaemic animals, we found that testosterone potentiated the training induced effects, and caused sparing of muscle glycogen after submaximal exercise. This appears to result from increased glycogen synthesis in combination with decreased glycogenolysis. Furthermore, the finding that glycogen sparing was significantly increased in trained animals may also be the result of an increase in the level of the skeletal muscle GLUT4 glucose transport protein. As glucose uptake by muscle cells is dramatically increased by insulin and by contractile activity itself, it is feasible that the glycogen sparing effect is caused by increased uptake through increased availability of GLUT4.\textsuperscript{30} Interestingly, Bruce et al\textsuperscript{39} reported that hexokinase and glycogen synthase activities are not altered in insulin resistant Zucker rats and suggested an identical flux through the GLUT4-hexokinase step driving the activation of glycogen synthase activity. In contrast with the above findings, the glycogen synthase activity in our hyperglycaemic animals was significantly lower, indicating a key difference between the models used. Therefore the hypothesis of Bruce et al\textsuperscript{39} that, in the face of muscle insulin resistance, glycogen supercompensation may occur after glycogen depleting exercise by increased glucose transport followed by increased hexokinase and glycogen synthase activity is not applicable to our model. Although their study was excellently carried out, the exercise model used (swimming) may put less stress on the muscle examined than our model (running). The glycogen sparing effects on endurance performance are profound. It would be interesting to determine whether glycogen sparing leads to improved performance, but was beyond the aim of our study. The only reliable marker, the time on the treadmill before exhaustion, is not a proven measure and is very difficult to assess in rats under such stressful circumstances. The same goes for other exercise regimens such swimming. Therefore the observed glycogen sparing effect during submaximal exercise after a prolonged period of training, as used in our study in streptozotocin hyperglycaemic animals with or without testosterone treatment, supports the hypothesis that regular training\textsuperscript{14} may counteract the cellular post-receptor defects in type 2 diabetes mellitus, and it may be argued that the combined metabolic defects observed during the late stages of type 2 diabetes mellitus can, in part, be counteracted by endurance training.

In addition to the above effects of training on glycogen synthesis, another major aim of this study was to investigate the effects of testosterone on glycogen metabolism. For instance, it has been shown that castration of male rats leads to hyperglycaemia and decreased glycogen stores accompanied by diminished glycogen synthase activity and increased glycogen phosphorylase activity.\textsuperscript{29} Interestingly, these impairments in metabolic variables remained within the normal range on testosterone treatment.\textsuperscript{29} On the other hand, Holmäng et al\textsuperscript{35} reported that, in ovariecotised female rats, hyperandrogenism impairs glucose disposal in skeletal muscle. We could not confirm this androgen induced insulin resistance in female rats, but the animals in our study were not ovariecotised.

The different responses of the soleus and EDL muscles are more difficult to explain but could be due to differences in
activity during running or the higher body weight of testosterone treated animals. This may also explain some of the differences between our study and that of Bruce et al., who used swimming as the exercise/training model. Furthermore, different expression of androgen receptors, a possible cause of different responses of different muscles, has been shown recently by Kadi et al. 22

Finally, comparison between studies is not easy. Differences in sex, surgery, levels of hormones administered, and exercise model used may be major causes of the observed discrepancies.

The present findings show that mild hyperandrogenicity affects muscle metabolism in mildly hyperglycaemic (first stage type 2 diabetic) female rats. Particularly in trained animals, we found that testosterone amplifies the training induced effects. This indicates that androgens are not, as suggested previously, a causative factor in the development of insulin resistance. Unravelling the exact mechanisms of the observed effects is a challenge for future investigations.

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