Effects of clenbuterol on insulin resistance in conscious obese Zucker rats

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Pan, Shujia J., Joe Hancock, Zhenping Ding, Donovan Fogt, Mancheong Lee, and John L. Ivy. Effects of clenbuterol on insulin resistance in conscious obese Zucker rats. Am J Physiol Endocrinol Metab 280: E554–E561, 2001.—The present study was conducted to determine the effect of chronic administration of the long-acting β2-adrenergic agonist clenbuterol on rats that are genetically prone to insulin resistance and impaired glucose tolerance. Obese Zucker rats (fa/fa) were given 1 mg/kg of clenbuterol by oral intubation daily for 5 wk. Controls received an equivalent volume of water according to the same schedule. At the end of the treatment, rats were catheterized for euglycemic-hyperinsulinemic clamp. Clenbuterol did not change body weight compared with the control group but caused a redistribution of body weight: leg muscle weights increased, and abdominal fat weight decreased. The glucose infusion rate needed to maintain euglycemia and the rate of glucose disappearance were greater in the clenbuterol-treated rats. Furthermore, plasma insulin levels were decreased, and the rate of glucose uptake into hindlimb muscles and abdominal fat was increased in the clenbuterol-treated rats. This increased rate of glucose uptake was accompanied by a parallel increase in the rate of glycogen synthesis. The increase in muscle glucose uptake could not be ascribed to an increase in the glucose transport protein GLUT-4 in clenbuterol-treated rats. We conclude that chronic clenbuterol treatment reduces the insulin resistance of the obese Zucker rat by increasing insulin-stimulated muscle and adipose tissue glucose uptake. The improvements noted may be related to the repartitioning of body weight between tissues.

skeletal muscle; glucose uptake; glycogen; triacylglycerol; adipose tissue; euglycemic-hyperinsulinemic clamp

CLENBUTEROL IS A LONG-ACTING selective β2-adrenergic agonist that is approved for human use outside the United States as a bronchodilator for the treatment of bronchial asthma. In animal studies with several species, evidence indicates that chronically administered clenbuterol can elicit an anabolic response in skeletal muscle (4, 29, 37). This anabolic effect is characterized by a significant increase in skeletal muscle protein as well as a decrease in body fat and is known as the “repartitioning” effect. The occurrence of this tissue redistribution is dissociated from any increase in food consumption by laboratory animals (28); however, there are few published data on the repartitioning effect in humans.

Interest in the human use of clenbuterol remains high because of its pronounced anabolic properties. In particular, its ability to change body composition and enhance muscle mass has led some to suggest that clenbuterol may have therapeutic potential for the treatment of obesity or metabolic disorders that are associated with obesity, such as type 2 diabetes.

Results pertinent to this issue have originated from research involving the obese Zucker rat. These animals are characterized by a genetic disorder that causes them to be obese and have abnormal glucose tolerance and that confers a marked insulin resistance. These properties make them a widely accepted model for the study of insulin resistance. Research has shown that chronic treatment of the obese Zucker rat with clenbuterol has a beneficial effect on its body composition (29, 35, 36). After treatment, the protein-to-fat ratio is increased by 173% due to a decrease in total body fat and an increase in skeletal muscle mass (28). Furthermore, a previous study from our laboratory (35) demonstrated an attenuation of insulin resistance in the obese Zucker rat after chronic treatment with clenbuterol, as evidenced by reductions in plasma insulin levels and improved glucose tolerance. Skeletal muscle is primarily responsible for glucose clearance during an oral glucose tolerance test or euglycemic clamp (5, 14). However, using the hindlimb perfusion procedure, we were unable to demonstrate an effect of clenbuterol on insulin-stimulated glucose uptake or transport in skeletal muscle (35, 36). This raised the question as to which tissues were responsible for the increased clearance of glucose in vivo and whether the hindlimb perfusion results could be extrapolated to the in vivo state. Therefore, the present study was conducted to determine more fully the effect of chronic administration of clenbuterol by use of the euglycemic-hyperinsulinemic clamp technique on the conscious obese Zucker rat. The
hypothesis tested was that the improvement in insulin resistance after chronic clenbuterol treatment is the result of an improved skeletal muscle response to insulin.

METHODS

Animal care and maintenance. Female obese (fa/fa) Zucker rats, 6–7 wk of age, were purchased from the Animal Model Core Facility of the University of California at Davis. The rats were housed in the Animal Resource Center of the University of Texas at Austin under standard laboratory conditions with access to food and water ad libitum. They were subjected to a controlled 12:12-h light-dark cycle, 22°C temperature, and 50% relative humidity. The rats were fasted for 12 h before the euglycemic-hyperinsulinemic clamp was performed. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Rats were assigned randomly to either a treatment or a control group. The treatment group was gavaged once a day to deliver clenbuterol at a concentration of 1 mg/kg body wt 7 days/wk for 5 wk. The clenbuterol treatment was stopped 48 h before the euglycemic-hyperinsulinemic clamp. The control group received only the equivalent volume of deionized water on the same schedule as the clenbuterol treatment group.

Catheterization. One day before catheterization, rats were placed in individual cages with the distal one-third of their tails secured by a rubber stopper through a hole at the back of the cage. This was done to accentuate the rats to this condition. The procedure was designed to protect catheters inserted into the tail veins and artery while allowing the rats nearly 180° mobility within the cage (38). The setup allowed free access to food and water and made it possible for intravenous infusions and blood withdrawals to be performed on conscious rats.

In preparation for infusion, catheters were inserted into both tail veins in the same manner as is ordinarily done for intravenous injections. A sampling catheter was also inserted into the tail veins and artery while allowing the rats to move freely within the cage (38). The setup allowed free access to food and water and made it possible for intravenous infusions and blood withdrawals to be performed on conscious rats.

Determination of blood [3-3H]glucose levels only. The sample at time 0 was considered to represent the basal sample before insulin infusion. After the 120-min blood sample was taken, [U-[14C]glucose (30 μCi) was administered as a bolus in <10 s duration by way of a tail vein catheter. This was done to measure glucose incorporation into glycogen. Blood samples (70 μl) were drawn 2, 5, 10, 15, 20, 30, and 45 min after the bolus injection for determination of [14C]glucose. When the clamp was completed, the rat was quickly anesthetized by an intravenous injection of pentobarbital sodium (50 mg/kg). Soleus, plantaris, red gastrocnemius, white gastrocnemius, red quadriceps, and white quadriceps from one leg, followed by the abdominal adipose tissues, liver, and heart, were removed and freeze-clamped as rapidly as possible with tongs cooled in liquid N2. All samples were stored at −80°C for later analyses of glycogen synthesis and GLUT-4 protein. Soleus and plantaris muscles were isolated in their entirety for determination of their weights. After these tissues were collected, the abdominal fat was excised and weighed. In brief, the stomach, intestines, and other visceral organs were removed, and carefully dissected of liver, myocardial and peritoneal fat. The kidneys were also excised and cleaned of visible fat. In addition, gonadal/inguinal fat, associated with the ovaries and fallopian tubes, as well as any fat remaining in the pelvic cavity, was removed. Finally, the surface of the abdominopelvic cavity, including the inferior surface of the diaphragm, and the body wall of the retroperitoneal and peritoneal spaces were carefully cleaned of visible fat.

Experiment B was designed to determine the glucose metabolic index (Rt) of different tissues and skeletal muscle fiber types as described by Kraegen et al. (17). The procedures of this experiment were the same as those described for Experiment A, except that these rats received a bolus injection of 2-deoxy-[2,6-3H]glucose (2-[3H]DG) instead of [14C]glucose, and during the euglycemic clamping no [3-3H]glucose tracer was added.

Analytical methods. Blood glucose concentrations were measured by a glucose oxidase method, using a One Touch blood glucose monitoring system (LifeScan, San Jose, CA). Plasma insulin levels during the euglycemic clamp were determined by a double antibody radioimmunoassay using the Linco RIA kit (Linco Research, St Louis, MO).125I radioactivity was measured with a Beckman 5500 γ-counter (Beckman, Fullerton, CA).

All blood samples of 2H and 14C were counted using a Beckman LS6000SC liquid scintillation spectrophotometer (Beckman, Fullerton, CA). The preparation for blood [3H]glucose measurement was as follows: 20 μl of blood sample were dissolved in 30% KOH and dried overnight to evaporate the tritiated water formed during metabolism of the tritiated glucose. The residue was resuspended in Bio-Safe II scintillation fluor (Research Products International, Mt. Prospect, IL) for radioactivity counting. The blood [14C]glucose measurement was performed by the same procedure as for [3H]glucose, except that there was no overnight drying process. The 2-[3H]DG blood sample was dissolved in 6% perchloric acid. After centrifugation, the supernatant was mixed with Bio-Safe II fluor for tritium counting. Glycogen synthesis was evaluated as the rate of [14C]glucose incorporated into glycogen in individual tissues. Tissue glycogen was isolated by the method of Lo et al. (21). Briefly, tissues other than adipose were solubilized in 30% KOH and saturated with Na2SO4 at 70°C, and glycogen was precipitated with 95% ethanol. Samples were then centrifuged, and the resultant glycogen pellet was resuspended in 0.9 ml H2O and 0.1 N HCl. A 0.4-ml aliquot of resuspended pellet was then added to 5 ml of Bio-Safe scintillation mixture and counted for
The glucose Rd was calculated by direct integration. When steady-state glucose infusion during the complete clamping period (165 min) was calculated by direct integration. When steady-state glucose infusion during the complete clamping period (165 min) was determined. Intramuscular triacylglycerol concentration was determined using a modified procedure of Frayn and Maycock (9). Briefly, 50 mg of frozen muscle were homogenized (glass-on-glass) in 3 ml of chloroform-methanol (2:1 vol:vol). Muscle lipid was extracted in the organic mixture after precipitation of nonpolar molecules with 3 ml of 4 mM MgCl₂. After centrifugation (1,000 g, 15 min) at 4°C for 1 h, the lipid-containing phase was evaporated to dryness with N₂ gas, leaving a small lipid pellet that was then resuspended with 6% perchloric acid for measurement of total 2-DG. The remaining part of the solution was then mixed with Ba(OH)₂-ZnSO₄ to precipitate the phosphorylated 2-DG. The remaining part of the solution was used to measure unphosphorylated 2-DG. After centrifugation, the supernatants were used to measure unphosphorylated 2-DG.

Immunohistochemical staining was used to demonstrate the presence of adipose tissue, muscle, liver, and heart. The GLUT-4 protein was assayed in red and white quadriceps muscle homogenates by use of quantitative Western blotting procedures, which involved digitizing autographs and using a heart reference standard, as reported previously (18).

Table 1. Body, organ, and tissue weights of obese Zucker rats

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Body Weight</th>
<th>Organ Weight</th>
<th>Tissue Weight</th>
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<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Liver</td>
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<td>g</td>
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<tr>
<td>Control</td>
<td>230</td>
<td>342</td>
<td>11.60</td>
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<tr>
<td>(n = 21)</td>
<td>±14</td>
<td>±6</td>
<td>±0.35</td>
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<tr>
<td>Clenbuterol</td>
<td>241</td>
<td>353</td>
<td>11.14</td>
</tr>
<tr>
<td>(n = 20)</td>
<td>±13</td>
<td>±6</td>
<td>±0.28</td>
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Values are means ± SE. *Significantly different from control (P < 0.05). †Abdominal adipose tissue. % body wt, percent body weight.
insulin in CT compared with control rats can account for this lower plasma insulin response.

Glucose metabolic rate. After 120 min of glucose and insulin infusion as described above, 2-[^3H]DG was administered as a bolus. The Rᵣ was then calculated in selected tissues and organs as described in METHODS. Application of this procedure is dependent on the 2[^3H]DG kinetics being fitted well by a biexponential curve. We found that the fit was excellent, with all r² values >0.98. An example of the curve fitting is illustrated in Fig. 4A. The Rᵣ was increased in the CT group relative to the control group in skeletal muscle and adipose tissue, but not in liver or heart (Fig. 5).

Fig. 1. Blood glucose and glucose infusion rate during euglycemic-hyperinsulinemic clamp. A: blood glucose levels over the course of 165 min for 21 control and 20 clenbuterol-treated (CT) obese Zucker rats. B: glucose infusion rate under the same conditions shown in A. Data points are expressed as means ± SE. *Significant difference from control.

Fig. 2. Rate of disappearance of glucose (Rᵣ); n = 11 for control group, and n = 10 for the CT group. Data are expressed as means ± SE. *Significant difference from control.

Fig. 3. Plasma insulin levels during euglycemic-hyperinsulinemic clamp; n = 21 for control group, and n = 20 for the CT group. Data are expressed as means ± SE. *Significant difference from control.

Fig. 4. Examples of data fitting using the biexponential model. A: biexponential model fitted to 2-deoxy-[2,6-[^3H]glucose (2[^3H]DG) data, with r² = 0.99. This set of 2[^3H]DG kinetic data was picked randomly from the CT group. B: biexponential model for D-[U-[^14C]glucose ([14C]glucose) fitted data, with r² = 0.99. This set of [14C]glucose data was selected randomly from the control rats.
With regard to the increase in skeletal muscle $R_g$, muscle composed of predominantly slow-twitch oxidative fibers, fast-twitch oxidative fibers, and fast-twitch glycolytic fibers all demonstrated a substantial improvement in insulin-stimulated $R_g$.

Glycogen synthesis. Glycogen synthesis was measured in the various tissues by the $R_g$ method described above. The kinetic data used in these calculations fit well by a biexponential curve, with $r^2$ values for all curves $>0.97$ and most being 0.99 (Fig. 4B). From these data, it was determined that the glycogen synthesis rates in the tissues investigated followed a pattern very similar to that of the $R_g$ (Fig. 6). The CT rats had glycogen synthesis rates that were significantly increased above the control in the adipose tissue and the three basic skeletal muscle fiber types. No differences in glycogen synthesis rates were found in the livers and hearts of CT and control rats. The only glycogen synthesis anomaly observed was that the rates of synthesis in the white gastrocnemii were not different for the CT and control rats.

GLUT-4 protein. No significant difference was observed between the GLUT-4 protein levels in the red and white quadriceps muscles of the CT rats relative to the GLUT-4 levels measured in the controls (Fig. 7A).

Triglyceride concentration. The intramuscular triglyceride concentration in red quadriceps muscles of the CT rats was significantly reduced compared with the control rats (Fig. 7B).

DISCUSSION

The major manifestations of insulin resistance are glucose intolerance and hyperinsulinemia, which re-
Clenbuterol reduces insulin resistance

The CT rats were accompanied by a lower plasma insulin response to glucose during euglycemic-hyperinsulinemic clamping (Fig. 3). This inverse relationship between glucose and plasma insulin levels suggests that the clenbuterol-induced increase in $R_d$ glucose was due to increased insulin action rather than to increased insulin secretion. This finding is in agreement with earlier studies in which chronic exposure to other β-adrenergic receptor agonists improved insulin action (13, 22, 31, 33). Lupien et al. (22), for example, found that chronic infusion of norepinephrine for 10 days resulted in a significant enhancement of glucose disposal during a euglycemic-hyperinsulinemic clamp in normal rats. Smith et al. (33) reported that the glucose tolerance and insulin action of the obese Zucker rat were significantly improved by chronic treatment with a novel β-adrenoceptor agonist. Moreover, Jacob et al. (13) recently reported that celiprolol, a β₁-antagonist/β₂-agonist, improved the glucose tolerance of the obese Zucker rat. In vitro insulin-stimulated glucose uptake of the fast-twitch epitrochlearis muscle was also improved. However, we have not been able to demonstrate, with an in vitro procedure, an enhanced insulin-stimulated glucose uptake in muscle of rats treated with clenbuterol, nor are we aware of any study that has investigated the fate of blood glucose in vivo after chronic treatment with this β₂-agonist.

In the present study, we demonstrated that the increase in insulin-stimulated glucose clearance in vivo after clenbuterol treatment occurred in all skeletal muscle fiber types and in abdominal adipose tissue (Fig. 4). In contrast, the effects of clenbuterol on glucose uptake by liver and heart tissues were insignificant. The increase in glucose uptake was paralleled by an increase in glycogen storage. In fact, 75–90% of the glucose taken up by skeletal muscle and adipose tissue was converted into glycogen. However, the rate of glucose uptake in adipose tissue was only a fraction of the rate of glucose uptake by skeletal muscle. Thus the major site of glucose clearance occurred in skeletal muscle, although adipose tissue may have contributed.

The increase in adipose tissue glucose uptake could be related to a reduced fat cell size. CT rats were found to have a significant reduction in abdominal adiposity, and this reduction was most likely due to a reduction in fat cell size rather than number (32). A strong inverse correlation between fat cell size and insulin sensitivity has been demonstrated (3). Consequently, an increase in fat cell insulin sensitivity could explain the increased rate of glucose uptake in the adipose tissue during the euglycemic-hyperinsulinemic clamp.

An explanation for the increase in insulin-stimulated skeletal muscle glucose uptake is not equivocal. It is of interest to note that the increase in muscle glucose uptake occurred in the absence of an increase in GLUT-4 protein concentration. In previous studies, we were unable to demonstrate that chronic administration of clenbuterol improved the skeletal muscle insulin sensitivity or responsiveness of the obese Zucker rat in vitro, although in vivo there were sub-

Fig. 7. A: GLUT-4 protein levels for red and white quadriceps expressed as a percentage of a rat heart standard. For both red and white quadriceps of control rats, n = 10, and for both muscles from clenbuterol-treated rats, n = 9. B: intramuscular triglyceride concentration of the red quadriceps; n = 12 and 11 for the control and clenbuterol-treated groups, respectively. *Significant difference between the two groups. Data are expressed as means ± SE.
stantial improvements in glucose tolerance and the insulin response to a glucose challenge (35, 36). Furthermore, we found that daily clenbuterol treatment prevents the exercise training-induced increase in skeletal muscle insulin responsiveness of the obese Zucker rat by attenuating the increased expression of GLUT-4 protein that normally occurs with exercise training (18). These results suggest the possibility that the improvement in skeletal muscle insulin action initiated by the clenbuterol treatment was systemic in nature. Therefore, a possible explanation for the difference in $R_d$ glucose between the CT and control rats could be related to differences in muscle blood flow.

The arteriovenous glucose difference and the rate of muscle blood flow determine muscle glucose uptake. Thus, with an increased skeletal muscle glucose uptake, blood flow could become rate limiting if glucose extraction were to exceed glucose delivery. Insulin stimulates increases in both skeletal muscle glucose extraction and blood flow in a dose-dependent manner (7, 19). Skeletal muscle blood flow is also under the influence of adrenergic regulation, of which $\beta_2$-adreceptors are the major adrenergic receptors of significance (11, 30). Although large increases in skeletal muscle blood flow have been demonstrated with acute administration of clenbuterol, chronic clenbuterol treatment was shown to reduce muscle blood flow (30). Therefore, the effect of clenbuterol on muscle glucose clearance mediated by increases in muscle blood flow may at best be transient and not maintained during chronic treatment (30).

Interestingly, chronic clenbuterol treatment has been found to elevate blood flow to white and brown adipose tissues while reducing blood flow to skeletal muscle (30). Consequently, sustained blood flow in adipose tissue may influence fat and energy metabolism in this tissue. An elevated lipolysis and thermogenesis may account for the reduction of abdominal fat in the CT rats and indirectly influence muscle tissue glucose uptake (6, 26).

Abdominal obesity is highly associated with insulin resistance. That is, the greater the abdominal adiposity, the greater the insulin resistance (1, 2, 16). Furthermore, reduction in abdominal fat is highly associated with improvement in whole body insulin action (6, 26). The mechanism by which abdominal obesity adversely influences insulin action and glucose tolerance is not known, but it may involve the overproduction of hormone-like substances such as tumor necrosis factor-α (TNF-α) from highly active adipocytes in the abdominal region. TNF-α levels are elevated in the obese Zucker rat (15). In vitro studies indicate that an excess of TNF-α will inhibit the tyrosine kinase activity of the insulin receptor (11), and chronic infusion of TNF-α will produce severe insulin resistance (23).

Abdominal obesity is also associated with an increase in intramuscular triglyceride stores, which have also been linked to insulin resistance (24). In the present study, the muscle triglyceride concentration of the control rats was substantially higher than is normally reported for nonobese rats (10). The intramuscular triglyceride concentrations of the CT rats, however, were 50% lower than those of the control rats. Although reduction in muscle triglyceride concentration may potentially explain the improvement in skeletal muscle insulin action, it does not address our previous finding that the improvement in muscle insulin resistance by clenbuterol treatment occurs only in vivo. If the improvement in insulin resistance was due to a lowering of the intramuscular triglyceride stores, it should also be detected in vitro as well as in vivo experiments. In addition, the reduction in intramuscular triglyceride concentration, although substantial, was not sufficient for normalization in the obese Zucker rat (10).

It should also be noted that the reduction in adiposity of the CT rats was accompanied by an increase in muscle mass. It is possible that this increase in muscle mass also contributed to the overall improvement in $R_d$ glucose during the euglycemic-hyperinsulinemic clamp. An increase in muscle mass could conceivably increase the available glucose storage area, thereby facilitating the clearance of glucose from the circulation. Several studies have documented improvements in the insulin response to a glucose challenge (25) and increase in $R_d$ glucose during a euglycemic-hyperinsulinemic clamp (24) when strength training increased fat-free mass. An increase in muscle mass, however, cannot account for the increase in muscle insulin action induced by the clenbuterol treatment.

In summary, the effect of chronic clenbuterol administration on insulin resistance of conscious, obese Zucker rats was assessed by the euglycemic-hyperinsulinemic clamp technique. In contrast to our previous in vitro findings, the present in vivo results demonstrate that the attenuation of insulin resistance by clenbuterol is accompanied by an increase in insulin-stimulated glucose uptake and glycogen synthesis in adipose and skeletal muscle tissues. This improvement in muscle insulin resistance occurred in the absence of an increase in GLUT-4 protein concentration. It is likely that this improvement in skeletal muscle insulin resistance is related to systemic changes possibly associated with changes in the body composition of the obese Zucker rat.

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REFERENCES


