Lowering Plasma Free Fatty Acids With Acipimox Mimics the Antidiabetic Effects of the β_3-Adrenergic Agonist CL-316243 in Obese Zucker Diabetic Fatty Rats

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We previously reported that long-term treatment of Zucker diabetic fatty (ZDF) rats with the selective β_3 agonist CL-316243 normalizes glycemia, decreases plasma free fatty acids (FFA) concentration, improves insulin responsiveness, and increases glucose uptake, not only in brown and white adipose tissues, but also in skeletal muscles. Because muscles do not express typical β_3 adrenoceptors, we postulated that the muscle effect was indirect and that it was possibly mediated by an activation of the glucose–fatty acid cycle. To test this hypothesis, we investigated the effects of Acipimox, a potent inhibitor of lipolysis in adipose tissue. Similar to CL-316243, Acipimox (150 mg/kg orally) markedly decreased plasma FFA, glucose, and insulin concentrations and improved glucose tolerance while reducing the insulin response in obese (350 to 400 g) ZDF rats. Plasma FFA concentrations were significantly correlated with plasma glucose and insulin concentrations ($r = .72$ and .83, respectively; $P < .01$), indicating strong metabolic relationships between these parameters. Euglycemic-hyperinsulinemic clamps combined with the 2-[^{3}H]deoxyglucose method revealed that Acipimox markedly improved insulin responsiveness and significantly increased glucose uptake (Rg′) in the diaphragm, the heart, and various skeletal muscles. Unlike CL-316243, Acipimox did not increase glucose use in brown or white adipose tissues. This selectivity shows that it is possible to improve diabetes in obese ZDF rats without necessarily stimulating thermogenesis in adipose tissues. Thus, decreasing plasma FFA with 2 drugs (Acipimox or CL-316243) that act via different mechanisms (acute inhibition of lipolysis or chronic stimulation of FFA oxidation) is associated with increased glucose uptake in muscles and enhanced insulin responsiveness. These observations support the hypothesis that CL-316243 may indirectly stimulate glucose uptake in muscles of type II diabetic rats by first stimulating brown adipose tissue (increasing uncoupling protein content and fatty acid oxidation) and progressively decreasing the levels of circulating FFA, resulting in activation of the glucose–fatty acid cycle or other mechanisms regulating insulin responsiveness in skeletal muscles.

WE RECENTLY reported that the selective β_3-adrenergic agonist CL-316243 normalizes glycemia, decreases insulinemia, decreases plasma free fatty acid (FFA) levels, increases insulin responsivenes, and markedly increases glucose uptake in peripheral tissues of obese Zucker diabetic fatty (ZDF) rats, a model of type II diabetes.[1] These rats develop frank diabetes at an early age (as early as 7 weeks), characterized by elevated plasma levels of glucose, insulin, FFA, triglyceride, and cholesterol.[1-3] At 10 weeks of age, blood insulin levels progressively decrease because of pancreatic β cell failure.[4-6] Similar to other rat models, such as the obese SHR rats, obese ZDF rats have several thermogenic and metabolic defects in their brown adipose tissue (BAT) that are reversed by long-term treatment with CL-316243.[1,7-9] Using euglycemic-hyperinsulinemic clamps combined with the 2-[^{3}H]deoxyglucose (2-[^{3}H] DG) method to estimate glucose use by peripheral tissues, we found that long-term treatment of obese ZDF rats with CL-316243 markedly improved insulin responsiveness (approximately 3-fold) and increased glucose uptake (Rg′) in BAT (21-fold), white adipose tissue (WAT; 2-fold), and skeletal muscles (2- to 3-fold) but not in the heart. In spite of the great increase of glucose uptake in BAT and WAT of obese rats, we estimated that glucose disposal in skeletal muscles was approximately 5-fold greater than in total adipose depots (BAT and WAT combined). Thus, even under adrenergic stimulation, the skeletal muscles still represented a more important site of glucose use than the total adipose tissue mass. This finding was somewhat unexpected because muscles, unlike adipose tissues, do not express β_3 adrenoceptors. Therefore, we postulated that stimulatory effects of CL-316243 on glucose uptake in muscles were indirect and that they were possibly mediated by the reduction in plasma FFA induced by CL-316243 treatment. According to Randle's hypothesis (the glucose–fatty acid cycle), such a reduction would enhance glucose use by skeletal muscles.[10] More recently it has been proposed that a reduction of lipid products in skeletal muscles might also increase insulin responsiveness.[11,12] Thus, we postulated that CL-316243 improves glucose use in diabetic rats by progressively improving the defective mitochondrial oxidative capacity of BAT and WAT, thereby increasing energy expenditure and fat oxidation and consequently reducing plasma FFA levels.[1,13] Decreasing plasma FFA with CL-316243 may lead to an enhancement of glucose use by skeletal muscles via the glucose–fatty acid cycle or other mechanisms, thereby contributing to normalization of glycemia.[11,12]

The principal goal of the present experiments was to test this hypothesis. We reasoned that if elevated levels of circulating FFA represent a major diabetogenic factor in obese ZDF rats, a decrease in FFA levels induced by means other than by CL-316243 treatment should also enhance glucose use by the skeletal muscles and decrease plasma glucose levels. We now report that a stable inhibitor of lipolysis in adipose tissues, Acipimox,[14-18] is effective in rapidly decreasing plasma FFA

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Submitted September 15, 2000; accepted January 18, 2001.

Supported by grants from the Canadian Diabetes Association and the Medical Research Council of Canada.

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0026-0495/01/5008-0002$35.00/0

doi:10.1053/meta.2001.24868

levels in obese ZDF rats. Remarkably, Acipimox treatment mimicked the beneficial effects of CL-316243 treatment on plasma glucose–FFA levels, insulin responsiveness, and glucose use by skeletal muscles but not by adipose tissues. This finding supports the hypothesis that the effects of CL-316243 on glucose use by skeletal muscles are indirectly mediated by its stimulatory effects on BAT thermogenesis (increase in uncoupling protein levels), resulting in increased FFA oxidation and decreased plasma FFA concentration.

MATERIALS AND METHODS

Animals and Treatments

Male ZDF rats (ZDF/Gmi-fa/fa) and their lean littermates were obtained from Genetic Models Inc (Indianapolis, IN) and were housed in individual cages at 23°C with a 12-hour light-dark cycle. The rats received Purina chow 5008 and water *ad libitum* and were used when they were 12 to 14 weeks old. The mean body weights of the lean and obese rats were 291.7 ± 4.1 g and 345.0 ± 7.9 g ($P < .01$).

Cannulations

Three to 4 days before the experiments, polyethylene cannulas filled with sterile heparinized saline (30 U/mL) were inserted under isoflurane anesthesia into the common carotid artery and / or the jugular vein (PE 10 or PE 50, respectively; Becton Dickinson, Parsippany, NJ), as previously described.[19] The cannulas were exteriorized through a neck incision, checked for patency, and sealed. All experiments were performed in unanesthetized, undisturbed, unrestrained rats 3 to 4 days after cannulation. By this time, the lean and obese rats had fully recovered from the cannulation: food intake, body weight gain, and plasma insulin and glucose levels had returned to precannulation values.

Acipimox Administration

A single dose of 3 mL of Acipimox (150 mg/kg; Pharmacia Upjohn, Milan, Italy) dissolved in saline was given orally to lean and obese ZDF rats via an animal-feeding needle. Control rats received the same volume of saline. After the gavage, we did not notice any sign of discomfort. During the tests, rats were lying quietly in their cages; they were unrestrained, and great care was taken to not disturb them.

Glucose Tolerance Tests

Glucose (0.5 g/kg) was injected into the common carotid artery via a PE 10 cannula in less than 30 seconds. The cannula was rapidly washed with saline, and arterial blood was sampled (0.2 mL) at various time points (see Fig 3). The samples were immediately replaced with an equivalent volume of saline. Blood samples were transferred into chilled heparinized tubes and centrifuged at 4°C, and the plasma was kept frozen (−80°C) for later insulin and glucose determinations. Total glucose and insulin areas under the curve were calculated by integrating the glucose and insulin values obtained during the entire 45 minutes of the tests.

Determination of Plasma Levels of Glucose, FFA, and Insulin

Plasma glucose levels were measured with a glucose analyzer (Beckman, Brea, CA). Insulin levels were determined by radioimmunoassay (Linco, St Charles, MO) using the conversion factor of 23.1 U/ng of rat insulin. Plasma FFA concentrations were determined using a nonesterified fatty acid kit (Wako Chemicals, Richmond, VA).

Determination of the Glucose Infusion Rate During Euglycemic-Hyperinsulinemic Clamps

Approximately 1 half hour before the experiment, polyethylene extension tubes were connected to the indwelling cannulas of the jugular vein (PE 50) and carotid artery (PE 10). A 4-way stopcock was used to infuse glucose, insulin, and radiolabeled tracers into the jugular vein, whereas the carotid artery was used for blood withdrawal. A first blood sample (10 μL) was taken and analyzed on the glucose analyzer. Then, 3 mL of Acipimox (150 mg/kg orally) dissolved in saline or 3 mL of saline (controls) was administered. After 1 hour, insulin (100 $mU \cdot kg^{-1} \cdot min^{-1}$) and glucose (1.66 mol/L) were infused in parallel. The rate of glucose infusion was adjusted to maintain euglycemia (7.1 to 8.3 mmol/L), and blood glucose concentration was tested at regular intervals.

Determination of the Rates of Glucose Uptake in Peripheral Tissues

Ninety minutes after starting euglycemic-hyperinsulinemic clamps, the rates of glucose uptake in peripheral tissues (Rg′) were estimated by the 2-[^{3}H]DG method,[20] using [U-^{14}C]sucrose as an extracellular marker.[1,19] In total, 15 samples of 10 μL were removed during clamping to check plasma glucose levels with a glucose analyzer and 7 samples of 100 μL were removed during the 2-deoxyglucose tests. The total blood volume removed (<1 mL) represents <5% of the total blood volume.

Statistics

The data were statistically analyzed using the unpaired *t* test or the paired *t* test when values from individual animals were compared before and after treatments. Correlations between variables were performed using least-squares regression analysis. Results are expressed as means ± SE.

RESULTS

Effects of Acipimox on Plasma Levels of FFA, Glucose, and Insulin in Lean and Obese ZDF Rats

Plasma FFA concentrations in unanesthetized, undisturbed ZDF rats were much higher in obese than in lean animals (Fig 1), confirming previous observations.[1-3] Oral administration of Acipimox (150 mg/kg) decreased the elevated plasma FFA levels of obese rats to approximately the same concentrations as in lean animals (about 0. 4 μEq/L). Preliminary dose-response experiments[21] showed that the dose of 150 mg/kg was optimal; larger doses did not further amplify Acipimox action. The decreased FFA levels remained unchanged for more than 3 hours. These initial observations confirmed that Acipimox is a much more stable inhibitor of lipolysis than its analogue, nicotinic acid, for which a rebound effect on plasma FFA levels is often observed.[14] The stability of the Acipimox effects allowed us to perform glucose tolerance tests and euglycemic-hyperinsulinemic clamping under near steady-state conditions (see below).

In addition to having higher plasma FFA levels, the obese rats were clearly diabetic (glycemia 14.53 ± 0.82 mmol/L) and hyperinsulinemic (insulinemia 0.74 ± 0.089 nmol/L); (Fig 1). In lean and obese rats, Acipimox treatment significantly reduced both plasma glucose and insulin concentrations. The effects of Acipimox on plasma FFA, glucose, and insulin concentrations were more evident in obese than in lean animals. Correlation analyses showed that the plasma levels of FFA in

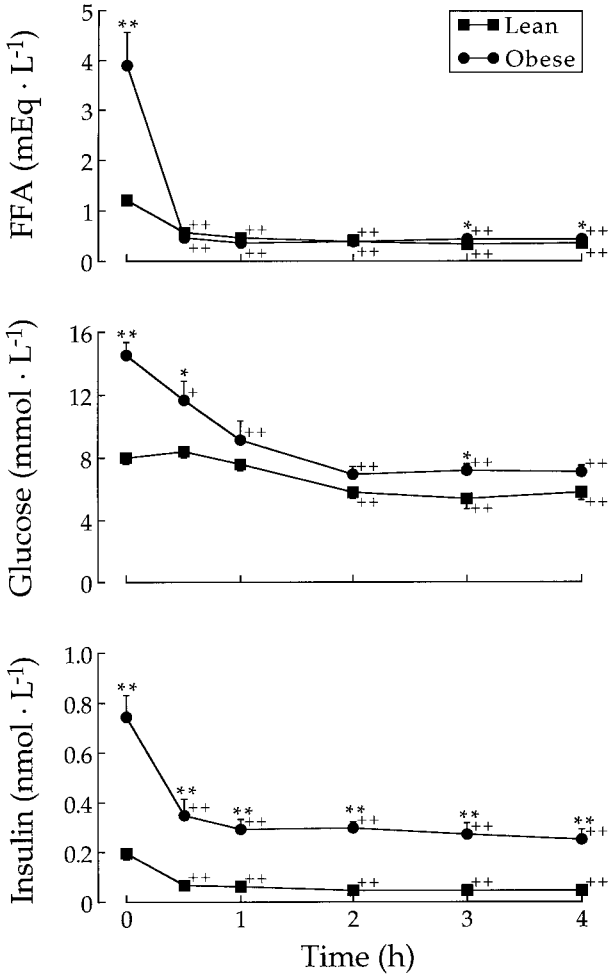

Fig 1. Effects of Acipimox on plasma FFA, glucose, and insulin levels in lean (n = 7) and obese (n = 6) ZDF rats. The rats were given a single dose of Acipimox (150 mg/kg orally) at time 0, and blood samples were removed and analyzed as described in the Methods. *,statistically significant differences between lean and obese animals: *$P < .05$, **$P < .01$. $^{+,++}$Statistically significant differences between values obtained immediately before (time 0) and after (4 hours) Acipimox injection: $^{+}P < .05$, $^{++}P < .01$.**

obese ZDF rats were highly correlated with the plasma levels of glucose ($r = .72$) and insulin ($r = .83$); ($P < .01$; Fig 2A and B).

Effects of Acipimox on Glucose Tolerance in Obese ZDF Rats

Acipimox treatment not only decreased glycemia and insulinemia in obese ZDF rats, but also significantly improved glucose tolerance (Fig 3). Confirming results shown in Figs 1 and 2, Acipimox significantly reduced the basal values (time 0) of plasma glucose and insulin. During the entire glucose tolerance test, both plasma glucose and insulin concentrations of treated rats remained decreased compared with concentrations in untreated animals (Fig 3A). Glucose did not significantly increase the elevated plasma insulin levels, indicating that the pancreatic β cells of obese rats were desensitized to glucose stimulation. The area under the glucose curve was decreased although the area under the insulin curve was also reduced (Fig 3B), an observation that supports increased insulin responsiveness, as also demonstrated by euglycemic-hyperinsulinemic clamping.

Effects of Acipimox on the Glucose Uptake of Obese ZDF Rats Under Euglycemic-Hyperinsulinemic Clamping Conditions

The next experiments were performed to test whether Acipimox improves insulin responsiveness in obese rats and to assess whether glucose use is modified by Acipimox in peripheral tissues such as the skeletal muscles, the heart, WAT, and BAT. For this purpose, we used the 2-[^{3}H]DG method for determining the rates of tissue glucose uptake (Rg′) under euglycemic-hyperinsulinemic clamping conditions. Unanesthetized precannulated obese rats were given either Acipimox (150 mg/kg orally) or the carrier solution and were left undisturbed for 1 hour to let the lipolytic inhibitor exert its effects (Fig 1). After this period, euglycemic-hyperinsulinemic clamping was performed as described in Materials and Methods. When steady-state conditions were reached, glucose and insulin con-

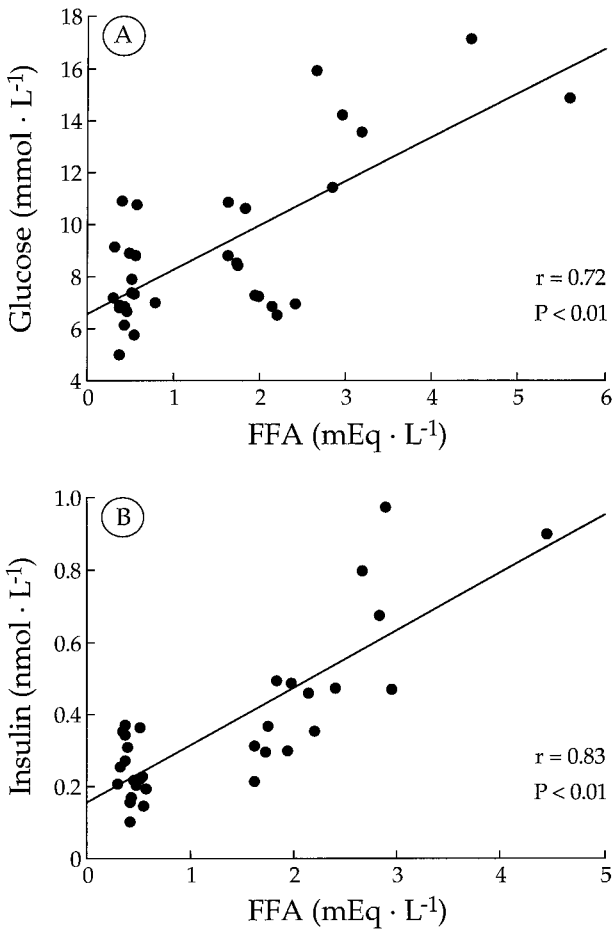

Fig 2. Correlation analyses between the concentrations of plasma FFA and (A) glucose or (B) insulin before and after Acipimox treatment. Values were taken from the experiment described in Fig 1.

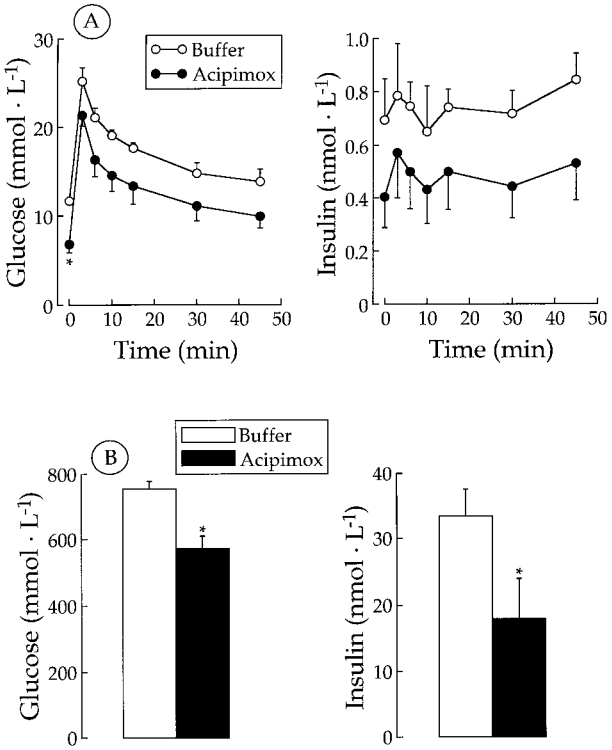

Fig 3. Effects of Acipimox on plasma glucose and insulin levels during a glucose tolerance test in obese ZDF rats. One group of obese rats (n = 5) received a single oral dose of Acipimox (150 mg/kg), and the control group (n = 3) was treated with the carrier solution. One hour later, the rats were injected with glucose (0.5 g/kg) via a preinstalled cannula, and plasma samples were taken at various times for analysis as described in the Methods. (A) Plasma glucose and insulin curves. (B) total glucose and insulin areas under the curves during the entire 45 minutes of the glucose tolerance test.

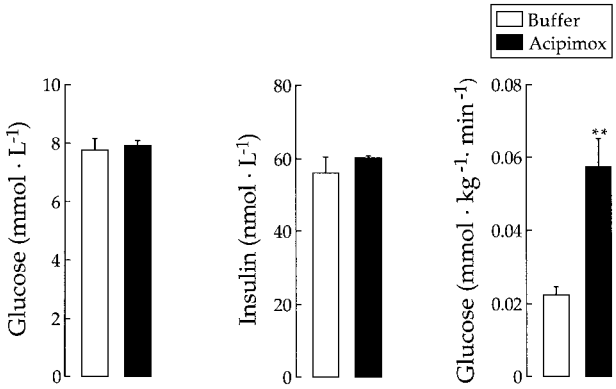

Fig 4. Glucose infusion rates during euglycemic-hyperinsulinemic clamping in obese ZDF rats treated with Acipimox. One group of obese rats (n = 5) received a single oral dose of Acipimox (150 mg/kg), and the control group (n = 4) was treated with the carrier solution. Euglycemic-hyperinsulinemic clamping and 2-[^{3}H]DG method were performed as described in the Materials and Methods. The data represent blood glucose levels (left), plasma insulin levels (middle), and glucose infusion rates (right) during clamping when steady-state conditions were reached.

centrations were similar in both groups (Fig 4). However, the amount of glucose infusion required to maintain euglycemia was 2- to 3-fold higher in Acipimox-treated rats than in untreated animals, indicating that the lipolytic inhibitor had significantly enhanced insulin action. Under these conditions, the 2-[^{3}H]DG tests showed that Acipimox significantly increased glucose uptake in various muscles (diaphragm, plantaris, and red and mixed gastrocnemius), in the heart, but not in BAT or WAT (Figs 5 and 6).

DISCUSSION

The main objective of this study was to test the hypothesis that the stimulatory effects of CL-316243 on glucose uptake in skeletal muscles of obese ZDF rats observed in previous studies[1] were indirectly mediated by a reduction in plasma FFA levels, leading to activation of the glucose–fatty acid cycle or other mechanisms modulating insulin responsiveness in skeletal muscles.[11,12] We were faced with the apparent paradox that a selective β_3 agonist such as CL-316243 significantly stimulated glucose uptake in skeletal muscles, a tissue that does not express typical β_3 adrenoceptors. We postulated that if CL-316243 acted indirectly on skeletal muscles by reducing plasma FFA levels, a similar reduction in FFA concentration obtained by other means, for instance by direct inhibition of lipolysis with Acipimox, should also enhance glucose use by the skeletal muscles. The present results support this hypothesis by showing for the first time that a reduction in plasma FFA concentration, directly obtained by inhibiting lipolysis with Acipimox, improves glycemia, increases insulin responsiveness, and stimulates glucose uptake in skeletal muscles of obese ZDF rats. Thus, short-term Acipimox treatment mimics many but not all metabolic effects induced by long-term CL-316243 treatment (see below).

The rapid and stable decrease in FFA levels induced by Acipimox was accompanied by decreases in both plasma glucose and insulin levels that apparently occurred after a delay of a few minutes (Fig 1). FFA concentrations before and after Acipimox injections were highly correlated with plasma glucose and insulin concentrations, indicating strong metabolic relationships between these parameters (Fig 2). Acipimox

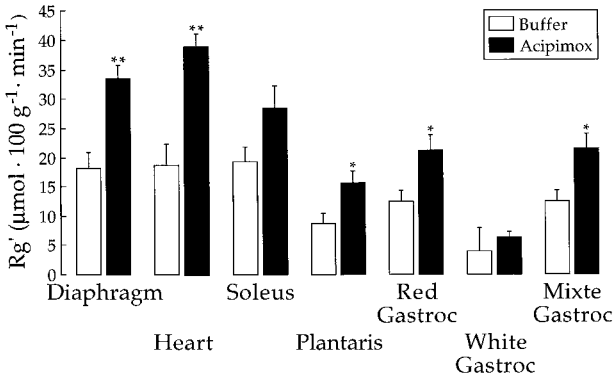

Fig 5. Effects of Acipimox treatment on the glucose uptake (Rg′) of various muscles (diaphragm; heart; soleus; plantaris; red, white, and mixed gastrocnemius).

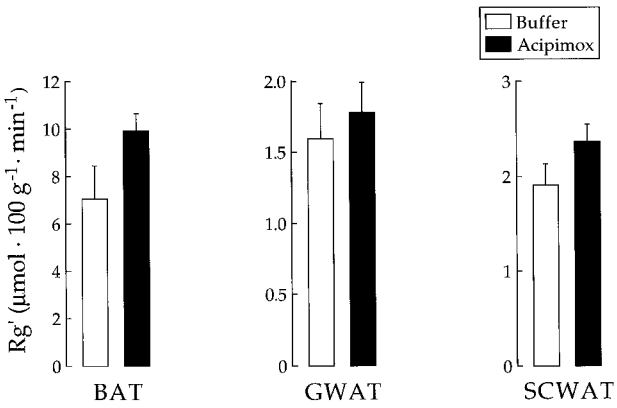

Fig 6. Effects of Acipimox treatment on the glucose uptake (Rg′) of interscapular BAT, gonadal WAT (GWAT), and subcutaneous WAT (SCWAT).

might have induced, directly or indirectly, other metabolic effects in diabetic rats. For instance, decreased FFA levels might also decrease hepatic glucose production or pancreatic insulin secretion, thereby contributing to lower plasma glucose and insulin levels. In any case, the present experiments show that Acipimox, similar to the β_3 agonist CL-316243, was able to induce a significant reduction in FFA in obese ZDF rats and that this phenomenon was highly correlated with decreases not only in glucose, but also in insulin concentrations (Figs 1 and 2). This finding strongly suggests that the Acipimox-induced decrease in FFA levels leads to stimulation of glucose uptake in skeletal muscle, resulting in decreased plasma glucose and insulin concentrations.

Glucose tolerance tests (Fig 3) showed that Acipimox improved glucose tolerance while reducing insulin response during an intravenous glucose tolerance test, a result that was also observed in our previous studies with CL-316243.[1] However, euglycemic-hyperinsulinemic clamping-clearly revealed important differences in the mode of action of Acipimox and CL-316243. Although both agents significantly increased glucose uptake in skeletal muscles, CL-316243, in contrast to Acipimox, markedly increased glucose uptake in BAT (21-fold) and WAT (approximately 2- to 3-fold) but not in the heart.[1] Assuming that the muscles, WAT, and BAT, respectively, represent 30%, 40%, and 1% of the body weight of obese rats, we calculated that glucose use in the entire brown and white adipose mass represents only 20% of that muscles (10% BAT and 10% WAT). Thus, muscles represent a much more important site of glucose use than adipose tissues, even when the latter are stimulated by β_3 agonists. The fact that Acipimox did not increase glucose uptake in adipose tissues also indicates that the effects of the lipolytic inhibitor are not mediated by activation of the sympathetic nervous system. Indeed, we previously reported that cold exposure, norepinephrine infusion, and CL-316243 treatment all increase glucose uptake in adipose tissues several-fold.[1,19,22] Glucose uptake in BAT is one of the best indicators of sympathetic activation because cold exposure (4°C) increases this parameter as much as 100-fold.

Both Acipimox and CL-316243 reduced the areas under the insulin and glucose curves during glucose tolerance tests, and both agents improved insulin responsiveness during euglycemic-hyperinsulinemic clamping (Figs 3, 4).[1] This finding indicates that during the glucose tolerance tests, the decrease in plasma insulin levels was more than compensated by an increase in insulin responsiveness, resulting in decreased areas under the glucose curves (Fig 3). A similar phenomenon occurs under more physiologic conditions, ie, during cold exposure in normal rats, and can be mimicked by infusing rats with norepinephrine, the physiologic effector of thermogenesis.[19,22,23]

The decrease in plasma FFA levels of obese ZDF rats was obtained in our previous studies by stimulating FFA oxidation in BAT, whereas in the present work, Acipimox inhibited lipolysis in WAT. Furthermore, Acipimox acted quickly (Fig 1), whereas 2 weeks of long-term treatment of obese rats with CL-316243 was required for obtaining similar results.[1] The explanation for the slow evolution of the antidiabetic effects of CL-316243 is based on the fact that thermogenesis in BAT of untreated obese animals is markedly defective. Total BAT mitochondrial content, cytochrome oxidase activity, and uncoupling protein (UCP 1) concentrations are all significantly reduced in rat models of obesity and type 2 diabetes.[1,7,8,24-26] Two weeks of treatment of obese animals with the β_3 agonist probably is required to stimulate mitochondrial proliferation and uncoupling protein concentration in BAT and to progressively increase thermogenesis in BAT.[1]

In summary, the principal similarities between the effects of Acipimox and CL-316243 on glucose metabolism in obese ZDF rats are that both drugs decrease plasma glucose, insulin, and FFA concentrations, improve insulin responsiveness, and increase glucose uptake in various muscles. The main differences are that Acipimox induces these effects quickly and does not stimulate glucose uptake in BAT or WAT, whereas CL-316243 exerts its antidiabetic effects only after several days of treatment and stimulates glucose uptake in adipose tissues, principally in BAT.[1] The observation that 2 drugs that decrease plasma FFA concentrations via 2 different mechanisms (inhibition of lipolysis or stimulation of thermogenesis) improve insulin resistance and enhance glucose uptake in muscles strongly suggests that mechanisms such as the classical glucose–fatty acid cycle or the regulation of glucose metabolism by intramuscular lipid products (such as long-chain acyl-CoAs)[11,12] are operative in muscles of ZDF rats. Further studies are needed to determine which of these mechanisms prevails in muscles of obese ZDF rats, but it is also possible that they function in parallel.

Finally, an important role for plasma FFA as a modulator of insulin action is also supported by numerous studies showing that increasing plasma FFA concentrations by various means (short-term infusions of FFA suspensions or of heparin with or without triglycerides) induces insulin resistance.[27] In fact, it is likely that the defective energy expenditure in obese ZDF rats caused by defective hypothalamic leptin receptors and sympathetic nervous system activity[28-30] gradually enhances WAT hyperthrophy-hyperplasia, which in turn causes increases in circulating FFA levels that progressively induce insulin resistance and diabetes. This progression can be temporarily reversed by CL-316243, which stimulates BAT thermogenesis and fat oxidation, bypassing the defective central sympathetic

stimulation, or more directly, by inhibition of FFA release from adipose tissues with Acipimox.

From a clinical point of view, each of these approaches has advantages. It is clear that β_3 agonists have the advantage of being antiobesity drugs that reduce adiposity by oxidizing fatty acids, a characteristic that is not shared by lipolytic inhibitors. Both types of drugs have been tested in recent clinical studies. Long-term treatment of healthy individuals with CL-316243 has been shown to increase insulin action and fat oxidation,[31] but similar studies of patients with type 2 diabetes still remain to be performed. New β_3 agonists, more specific for human β_3 adrenoceptors (which are different from rat β_3 adrenoceptors), are presently being tested.[32] On the other hand, it has been reported that Acipimox acutely improves glucose tolerance and insulin resistance in type 2 diabetics[33-35] and ameliorates glucose tolerance in hyperthyroid patients.[36] Using the 2-[^{3}H]DG technique, we were able to confirm and extend these observations by directly demonstrating that Acipimox increases glucose uptake in different types of skeletal muscles of type 2 diabetic rats without affecting glucose use in BAT or WAT. To our knowledge, the long-term effects of drugs stimulating fatty acid glucose lipolysis uptake have not yet been evaluated in humans or laboratory animals. More studies, particularly long-term clinical studies, are needed to determine which of these approaches would be more effective for treatment of type 2 diabetes.

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