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Evidence for Posttranscriptional Regulation of GLUT4 Expression in Muscle and Adipose Tissue from Streptozotocin-Induced Diabetic and Benfluorex-Treated Rats

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ABSTRACT. In this study we explored the expression of GLUT4 glucose carriers in muscle and adipose tissues from streptozotocin-induced diabetic and benfluorex-treated rats. In nondiabetic rats, benfluorex treatment decreased GLUT4 protein content in muscle and brown adipose tissue, with no change in GLUT4 mRNA. This effect occurred in the presence of normal circulating levels of insulin and glucose. Seventeen days after streptozotocin injection, diabetic rats showed a decreased GLUT4 protein content in adipose tissues and in both red and white skeletal muscle. Diabetic rats showed decreased GLUT4 mRNA levels in white and brown adipose tissue, whereas messenger concentrations remained unaltered in red and white fibers of skeletal muscle. The interaction of benfluorex and diabetes on GLUT4 protein expression showed a tissue-specific pattern. Benfluorex treatment to some extent prevented the decrease in GLUT4 protein in white and brown adipose tissue and in white muscle associated with diabetes. In contrast, diabetes and benfluorex caused an additive decrease in GLUT4 expression in red skeletal muscle. The effects of benfluorex on GLUT4 content in tissues from diabetic rats occurred in the absence of alterations in GLUT4 mRNA levels, suggesting a modification of translational or posttranslational steps. Benfluorex did not ameliorate the hyperglycemia of diabetic rats. Our results indicate that red and white skeletal muscle respond to diabetes and benfluorex in a heterogeneous manner, which suggests the existence of differences in the mechanisms that regulate GLUT4 expression. Furthermore, our data indicate that GLUT4 expression in muscle and adipose tissue can be regulated by modification of translational or posttranslational steps. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1665–1673, 1996.

KEY WORDS. benfluorex; streptozotocin-induced diabetes; glucose transporters; skeletal muscle; white adipose tissue; brown adipose tissue; in vivo

Tissues that acutely respond to insulin by increasing glucose uptake show a unique feature; they mainly express the glucose carrier isoform GLUT4 [1, 2]. In fact, GLUT4 transporters represent the vast majority of glucose carriers expressed in rat adipocytes and skeletal muscle [3, 4]. Under basal conditions, GLUT4 is mainly intracellular, and insulin causes the rapid translocation of GLUT4-containing vesicles from an intracellular site to the cell surface [1, 2]. The expression of GLUT4 is highly regulated in adipose tissue and muscle. Diabetes and fasting drastically decrease GLUT4 protein and mRNA levels in white adipose tissue [5–9] due to a diminished transcriptional activity [10]. GLUT4 protein, RNA levels and GLUT4 gene transcription have been reported to decrease in skeletal muscle from streptozotocin-induced diabetic rats [7, 11–14]. However, a more complex pattern of alterations in muscle GLUT4 expression have been reported in some insulin-resistant states. Thus, under some conditions, decreased GLUT4 protein levels in skeletal muscle are concomitant with normal concentrations of GLUT4 mRNA [9, 15]; under some other conditions, muscle GLUT4 mRNA levels are modified in the absence of changes in GLUT4 protein content [9, 16, 17]. These observations suggest the existence of regulation of GLUT4 expression at translational or posttranslational steps.

Benfluorex [1-3-(trifluoromethylphenyl)-2-(2-benzoyloxyethyl) aminopropane hydrochloride] is used as a hypolipidemic and antihyperglycemic agent [18–21]. It inhibits the synthesis of triacylglycerol and cholesterol [22–24] and the release of VLDL† by the liver [25]. In addition, it may lower blood glucose under certain conditions characterized by insulin resistance. Thus, benfluorex has a direct

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† *Abbreviations:* SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; VLDL, very low-density lipoproteins.

inhibitory effect on gluconeogenesis from different precursors in isolated rat hepatocytes [25], and chronic benfluorex treatment normalizes hyperglycemia and reverses hepatic insulin resistance in a rat model of noninsulin dependent diabetes mellitus that is induced by injection of streptozotocin 5 days after birth [26]. Chronic administration of benfluorex improves the sensitivity of glucose utilization to insulin and lowers plasma insulin concentrations in several models of insulin resistance, such as fat feeding, fructose feeding and genetic obesity [27–29]. The fact that benfluorex ameliorates the insulin resistance of hepatic and peripheral tissues raises the question as to whether benfluorex treatment modifies the expression of glucose transporters in those tissues. As a first attempt to characterize the antidiabetogenic properties of benfluorex, we have studied the effect of chronic treatment with benfluorex on metabolic parameters and GLUT4 glucose transporter expression in muscle and adipose tissue from nondiabetic and streptozotocin-induced diabetic rats. Our studies show that GLUT4 expression is frequently regulated via posttranscriptional mechanisms in muscle and adipose tissue.

MATERIALS AND METHODS

Materials

Benfluorex was obtained from the Institut de Recherches Internationales Servier (Courbevoie Cedex, France). [^{125}I]-protein A, [^{32}P]-deoxy-cytidine triphosphate and Hybond N were purchased from Amersham (Buckinghamshire, UK). Random priming DNA labeling kit was obtained from Boehringer Mannheim (Mannheim, Germany). Immobilon was obtained from Millipore (Bedford, MA, USA). All electrophoresis reagents, molecular weight markers and reagents for protein assay were obtained from Bio-Rad (Hercules, CA, USA). Gamma-globulin and most commonly used chemicals were obtained from Sigma (St. Louis, MO, USA).

Animals and Treatment

Male Sprague-Dawley rats (180–200 g) obtained from Panlab were used. The rats were fed on Purina Laboratory chow ad libitum and housed in animal quarters maintained at 22°C with a 12-hr light, 12-hr dark cycle. Each cage contained three rats. Diabetes was induced by an intraperitoneal injection of streptozotocin (65 mg/kg b.w.). Benfluorex was administered orally at a dose of 35 mg/kg b.w. in both control and diabetic rats between 9 and 11 a.m. Benfluorex treatment was initiated 3 days after streptozotocin injection and was maintained for 2 weeks. The benfluorex was suspended at 7.5 mg/mL in 0.5% (w/v) arabic gum in water, and control rats were given the equivalent volume of vehicle. The rats were weighed, and food and water intake for each cage were measured daily.

Tissue Sampling

At different days throughout the experiment, blood was drawn from the tail and placed in heparinized tubes. Two weeks after benfluorex administration, a sample of blood was obtained from the tail, and the animals were then anesthetized with sodium pentobarbital (5–7 mg/100 g b.w.), and interscapular brown adipose tissue, epididymal fat pads, heart and red and white skeletal muscle were rapidly excised. Red muscle consisted of pooled red portions of the gastrocnemius and quadriceps muscles, and, similarly, white portions of the gastrocnemius and quadriceps were pooled as the source of white muscle. Based on previous studies documenting fiber composition in different muscles of the rat [30, 31], the red muscle fraction consists primarily of fast-twitch oxidative fibers and the white muscle largely of fast-twitch glycolytic fibers. After collection, tissues were rapidly frozen and kept in liquid nitrogen until analysis. The deproteinized filtrates of plasma were neutralized with 0.5 M KOH/triethanolamine. Glucose and β-hydroxybutyrate were determined by standard enzymatic methods as previously described [32]. Plasma samples were used for determination of insulin by radioimmunoassay.

Preparation of Total Membrane Fractions from Tissues

Total membrane fractions were obtained as previously described [9, 17]. In short, tissues were homogenized with Polytron (setting 6, 2 × 30 s) in 10 vol ice-cold buffer containing 25 mM Hepes, 250 mM sucrose, 4 mM EDTA, 1 trypsin inhibitory unit/mL aprotinin, 25 mM benzamidine, 0.2 mM PMSF, 1 μM leupeptin and 1 μM pepstatin, pH 7.4. Homogenates from brown and white adipose tissue and heart were centrifuged at 5000*g* for 5 min at 4°C. The supernatant was then centrifuged at 150,000*g* for 2 hr at 4°C to obtain the membrane fractions. The homogenates from red and white muscle were centrifuged at 15,000*g* for 20 min at 4°C. The supernatants were adjusted to 0.8 M KCl, incubated at 4°C for 30 min, and then centrifuged for 90 min at 200,000*g* at 4°C to obtain the total membrane fractions. The membrane pellets were resuspended in homogenization buffer and repeatedly passed through a 25-gauge needle before storage at −20°C. Proteins were measured by the method of Bradford [33] by using gammaglobulin as a standard.

Electrophoresis and Immunoblotting of Membranes

SDS-PAGE was performed on membrane protein in accordance with the method of Laemmli [34]. Proteins were transferred to Immobilon, as previously reported [9], in buffer consisting of 20% methanol, 200 mM glycine and 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5% nonfat dry milk in 0.02% sodium azide in PBS for 1 hr at 37°C and then incubated with polyclonal antibody OSCRX raised against the ^{15}C-terminal peptide from GLUT4 for the same time and at the same temperature. Polyclonal antibody OSCRX specifically recognizes GLUT4 [17, 35]. Transfer was confirmed by Coomassie blue staining of the gel after the electroblotting. Antibody OSCRX purified by protein A chromatography was used at 5–10 μg/mL in 1% nonfat dry milk/0.02% sodium azide in

PBS for immunoblotting. Detection of antibody–antigen complexes were effected with ^{125}I-protein A and autoradiography. The autoradiograms were quantified by using scanning densitometry (Ultrascan × L enhancer laser densitometer, LKB). Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range, and data were expressed as a percentage of control values.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from heart, epididymal white adipose tissue and red and white muscle by using the acid guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi [36]. All samples had a 260:280 absorbance ratio above 1.7.

After quantification, total RNA (15–30 μg) was denatured at 65°C in the presence of formamide, formaldehyde and ethidium bromide [37] to allow the visualization of RNA. RNA was separated on a 1.2% agarose/formaldehyde gel and blotted on Hybond N filters. The RNA in gels and filters was visualized with ethidium bromide and photographed by UV transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of total RNA and to confirm proper transfer. RNA was transferred in 10× standard saline citrate (SSC; 0.15 NaCl and 0.015 M sodium citrate, pH 7.0).

Blots were initially prehybridized for 4 hr at 45°C in 50% formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrolidone, 0.02% Ficoll, 0.02% BSA), 0.5% SDS, 5× SSPE (1× SSPE is 0.15 M NaCl, 1 mM EDTA and 10 mM NaH_2PO_4, pH 7.4) and 100 μg/mL of denatured salmon sperm DNA. The blots were then hybridized to the corresponding probes for 12 hr at 42°C in 50% formamide, 5× Denhardt's solution, 0.5% SDS, 5× SSPE, 10% dextran sulphate and 100 μg/mL of denatured salmon sperm DNA. The human cDNA probe for GLUT4 is a 2007-base-pair SalI fragment, which was obtained from Dr. Graeme I Bell (University of Chicago). The genomic probe for β-actin is a 4500-base-pair HindIII and EcoRI fragment [38]. The DNA probes were labelled with [^{32}P]-deoxy-CTP by random oligonucleotide-priming. The probes were included at 1.5×10^6 cpm/mL. Filters from GLUT4 assays were washed for 15 min in 2× SSC at room temperature and then twice in 0.4× SSC, 0.1% SDS (first wash for 20 min and second wash for 30 min) at 55°C. The abundance of specific glucose transporter message was quantified by scanning densitometry of autoradiograms as described above, and data were expressed as a percentage of control values. Analysis of variance or Student's t tests was used for statistical analysis.

RESULTS

General Observations and Metabolic Parameters in Streptozotocin-Induced Diabetic and Benfluorex-Treated Rats

Benfluorex administration (35 mg/kg b.w./day) to normal rats was followed by a rapid and substantial reduction in food intake. Although food intake on day 1 of treatment was markedly reduced (51%), it partially recovered, and on days 7 and 14, the reduction in food intake was 27% and 16%, respectively. In parallel, benfluorex treatment also caused a decreased water intake, and on day 14 of treatment the reduction was approximately 17%. The treatment of normal rats with benfluorex also led to a reduction in body weight. As a result, 14 days after treatment, rats in the benfluorex group had gained only 79 g, whereas controls gained 129 g, a decrease of 39%. Similar effects of benfluorex have been reported for food intake and body weight gain in JCR:LA corpulent rats [22]. Moreover, a 46% decrease in weight of epididymal adipose fat was observed in benfluorex-treated rats, whereas no significant change was noted in the weight of soleus muscle or interscapular brown adipose tissue (data not shown). The concentration of plasma glucose was determined in control and benfluorex-treated rats at different days throughout the experiment (on days 3, 7, 11 and 14 of treatment). Benfluorex did not modify plasma glucose levels at any time of the treatment (data not shown). After 2 weeks of treatment, control and benfluorex groups showed similar concentrations of circulating insulin and of plasma β-hydroxybutyrate (data not shown).

Diabetes was induced by administration of a single dose of streptozotocin (65 mg/kg b.w. on day −3 of the experimental protocol), which led to a rapid and steady decrease in body weight gain and an increase in food intake and water consumption (data not shown). However, the maximum effect of streptozotocin on food intake was only detected 8 days after its injection (data not shown). Seventeen days after streptozotocin injection, diabetes reduced the weight of soleus muscle (130 ± 5 and 105 ± 5 mg in control and diabetic rats, respectively), interscapular brown fat (281 ± 42 and 172 ± 21 mg in control and diabetic rats, respectively) and epididymal adipose fat (2.3 ± 0.3 and 2.3 ± 0.4 g in control and diabetic rats, respectively). Benfluorex treatment started 3 days after streptozotocin injection, when rats had already developed marked hyperglycemia. Food intake initially decreased in diabetic rats treated with benfluorex on day 1 (42% decrease) and day 2 (25% decrease) of treatment. However, this effect was somewhat reduced after day 4 of benfluorex administration (data not shown). Results of water consumption were recorded from day 6 to day 14 of benfluorex treatment. As observed in nondiabetic rats, benfluorex slightly reduced the water intake and also lowered the rate of body weight gain in diabetic rats (data not shown). Thus, benfluorex-treated diabetic rats gained only 32 g, whereas untreated rats gained 47 g, a 32% decrease. Injection of streptozotocin to rats caused the rapid development of hyperglycemia. Twenty-four hours after streptozotocin administration, plasma glucose levels were already high (Fig. 1) and remained very high throughout the experiment. Treatment of diabetic rats with benfluorex did not significantly modify the pattern of plasma glucose concentrations (Fig. 1). After 2 weeks of benfluorex, diabetic rats displayed high concentrations of

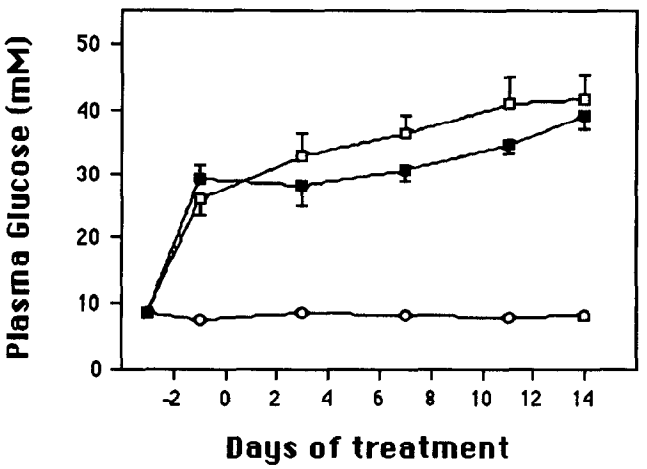

FIG. 1. Effect of benfluorex and diabetes on plasma glucose concentrations. Rats were injected with streptozotocin at day -3. On day 0, benfluorex treatment was initiated. At different times of the experimental protocol, blood samples were obtained from untreated nondiabetic (circle), untreated diabetic (open square) and benfluorex-treated diabetic (solid square) rats, and plasma glucose was assayed by enzymatic analysis. Results are means ± SE of 5–10 observations per group. Differences between benfluorex-treated and untreated groups were statistically insignificant.

β-hydroxybutyrate and low levels of plasma insulin, and benfluorex did not modify the plasma concentrations of these two parameters (data not shown).

Expression of GLUT4 Protein in Tissues from Streptozotocin-Induced Diabetic and Benfluorex-Treated Rats

After 2 weeks of benfluorex treatment, we determined the tissue content of GLUT4 in muscle and adipose tissue from nondiabetic rats. To this end, total membrane fractions were purified from epididymal and interscapular adipose tissues, and red and white skeletal muscle was obtained from control and benfluorex-treated rats. No difference was detected in the yield of membrane proteins obtained per gram of tissue in any of the tissues studied (data not shown). Treatment with benfluorex caused a significant decrease in the total content of GLUT4 protein in red muscle (30%), white muscle (47%) and brown adipose tissue (38%) from nondiabetic rats (Figs. 2, 3). No significant difference in the content of GLUT4 protein was detected in white adipose tissue from normal rats in response to benfluorex (Fig. 3). This overall pattern of changes in GLUT4 protein content was evident when data were expressed either per microgram of protein or per gram of tissue (data not shown).

Diabetic rats showed a substantial decrease in the content of GLUT4 protein in red muscle (49%), white muscle (62%), white adipose tissue (78%) and brown adipose tissue (77%) when studied 17 days after streptozotocin administration (Figs. 2, 3). The interaction of benfluorex and diabetes on GLUT4 expression showed a complex pattern. Benfluorex treatment for 2 weeks largely prevented the de-

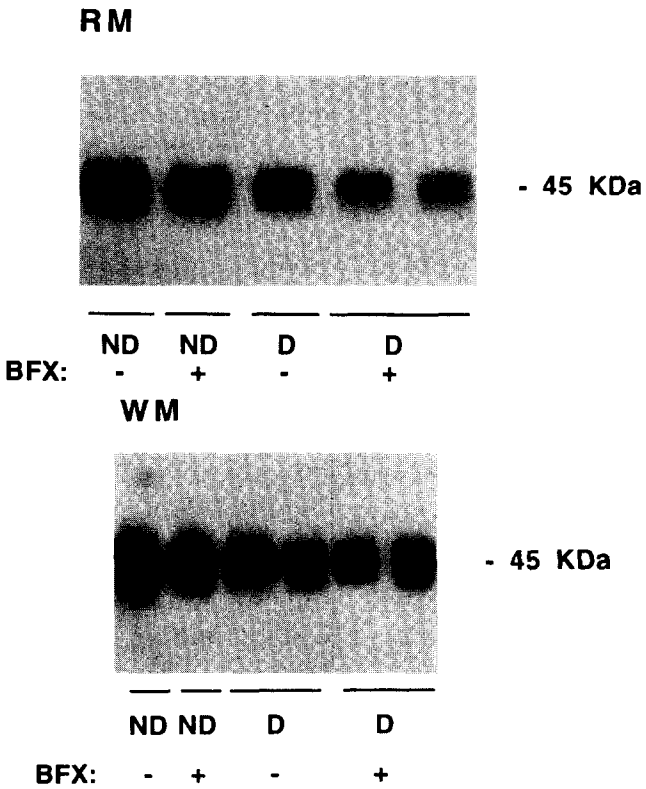

FIG. 2. Effect of benfluorex and diabetes on GLUT4 protein content in muscle. Rats were injected with streptozotocin at day -3 of the experimental protocol. On day 0, benfluorex treatment (35 mg/kg b.w.) was initiated. On day 14 of benfluorex treatment, untreated (-) or benfluorex-treated (+) nondiabetic (ND) or diabetic (D) rats were killed and tissues collected and processed; 20 μg of proteins from red (RM, top) and white muscle (WM, bottom) membrane proteins was applied on gels for Western blots. GLUT4 protein was detected by incubation with polyclonal antibody OSCRX. Representative autoradiograms obtained after different times of exposure are shown.

crease in GLUT4 protein content in white and brown adipose tissue associated with diabetes (Figs. 2, 3). Thus, GLUT4 protein levels in white and brown adipose tissue from benfluorex-treated diabetic rats only decreased by 13% and 32% as compared with values detected in benfluorex-treated non-diabetic rats (Fig. 3). As a result, benfluorex treatment in diabetic rats led to higher GLUT4 protein levels in brown and white adipose tissues than in the untreated diabetic group (Fig. 3). Streptozotocin-induced diabetes or benfluorex treatment alone caused a marked reduction in GLUT4 protein content in white skeletal muscle (Fig. 2); however, no differences in GLUT4 protein were found in white muscle when comparing the benfluorex-treated nondiabetic and the benfluorex-treated diabetic groups, suggesting a protective effect of benfluorex against the effect of diabetes. In contrast with these observations, diabetes and benfluorex caused an additive reduction in GLUT4 protein content in red skeletal muscle (Fig. 2). This pattern of changes in GLUT4 protein content occurred under conditions in which the yield of membrane

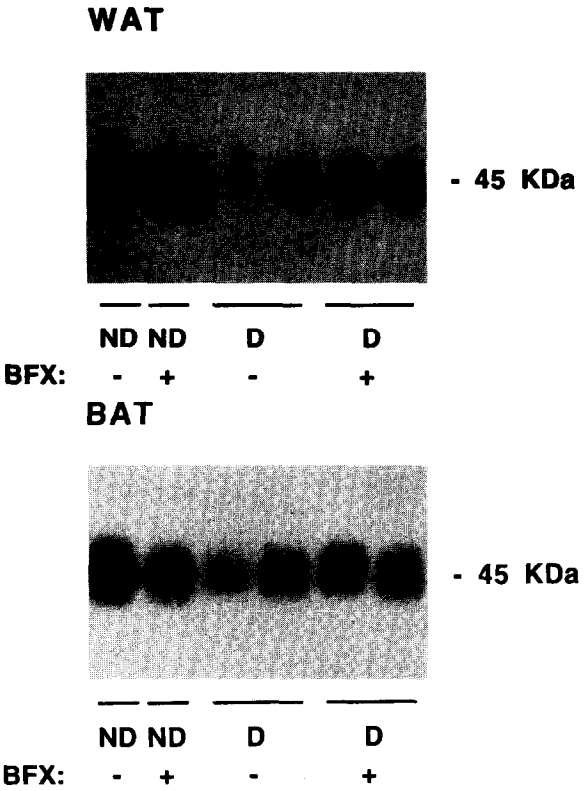

FIG. 3. Effect of benfluorex and diabetes on GLUT4 protein content in adipose tissues. Rats were injected with streptozotocin at day -3 of the experimental protocol. On day 0, benfluorex treatment (35 mg/kg b.w.) was initiated. On day 14 of benfluorex treatment, untreated (-) or benfluorex-treated (+) nondiabetic (ND) or diabetic (D) rats were killed and tissues collected and processed; 10 μg of white adipose (WAT, top) and brown adipose tissues (BAT, bottom) membrane proteins was applied on gels for Western blots. GLUT4 protein was detected by incubation with polyclonal antibody OSCRX. Representative autoradiograms obtained after different times of exposure are shown.

proteins per gram of tissue was similar for all tissues studied (data not shown).

Expression of GLUT4 mRNA in Tissues from Streptozotocin-Induced Diabetic and Benfluorex-Treated Rats

To determine the basis for the alterations in glucose transporter expression in muscle tissues from nondiabetic and diabetic rats in response to benfluorex treatment, we next determined the tissue levels of GLUT4 mRNA. Total RNA was purified from tissues obtained from control and benfluorex-treated rats. No difference was found in the yield of total RNA obtained per gram of tissue in control and benfluorex-treated nondiabetic rats (data not shown). Levels of mRNA for GLUT4 in adipose tissue and all muscles studied were indistinguishable when benfluorex-treated and untreated nondiabetic rats were compared (Figs. 4, 5). This pattern was evident when data were expressed as per microgram of total RNA, per unit of β-actin mRNA levels or per gram of tissue (data not shown). Thus, the reduction in the content of GLUT4 protein noted in the benfluorex-treated nondiabetic group was not explained by a concomitant decrease in the levels of GLUT4 mRNA.

We also studied the effects of diabetes and of benfluorex treatment on GLUT4 mRNA levels in skeletal and adipose tissues. A decrease of approximately 20–25% in the yield of total RNA was observed in red and white muscle in response to diabetes, and this was not affected by benfluorex treatment (data not shown). Diabetes caused a large decrease in GLUT4 mRNA concentrations in white and brown adipose tissues (Fig. 5); β-actin mRNA levels in adipose tissues also markedly decreased in response to diabetes (data not shown). In contrast, GLUT4 and β-actin mRNA levels remained unaltered in red or white skeletal muscle from diabetic rats (Fig. 4). Benfluorex did not alter GLUT4 mRNA levels in muscle or adipose tissues from untreated diabetic rats (Figs. 4, 5).

Table 1 shows the GLUT4 mRNA:GLUT4 protein ratio found in adipose and muscle tissues under the four conditions subjected to study. Benfluorex or diabetes caused a marked increase in the GLUT4 mRNA/protein ratio in red and white skeletal muscle (Table 1). Diabetes or benfluorex also enhanced the GLUT4 mRNA/protein ratio in brown adipose tissue; however benfluorex treatment in diabetic rats decreased the mRNA:protein ratio (Table 1). A similar pattern was also observed in white adipose tissue (Table 1).

DISCUSSION

In this study, we have made the following observations: (a) GLUT4 protein diminished in skeletal muscle from diabetic rats 17 days after streptozotocin administration and in

TABLE 1. Effect of diabetes and benfluorex on the GLUT4 mRNA:GLUT4 protein ratio in muscle and adipose tissues

	Control	Benfluorex	Diabetes	Diabetes + Benfluorex
Red muscle	1.0	1.34 ± 0.16	1.67 ± 0.32*	2.80 ± 0.34#
White muscle	1.0	2.14 ± 0.19*	2.19 ± 0.22*	2.07 ± 0.21
White adipose tissue	1.0	1.29 ± 0.23	2.89	0.71
Brown adipose tissue	1.0	1.89 ± 0.30*	1.87 ± 0.26*	0.99 ± 0.16#

GLUT4 mRNA content expressed as units/μg of RNA and GLUT4 protein expressed as units/μg of protein (data from Figures 2 and 6) were recalculated as a ratio of GLUT4 mRNA to GLUT4 protein (mean ± SE). Ratios in diabetic and benfluorex-treated groups were expressed as percentages of control values.

* Significant difference between control and experimental groups at $P < 0.05$.

\# Significant difference between untreated and benfluorex-treated diabetic rats at $P < 0.05$.

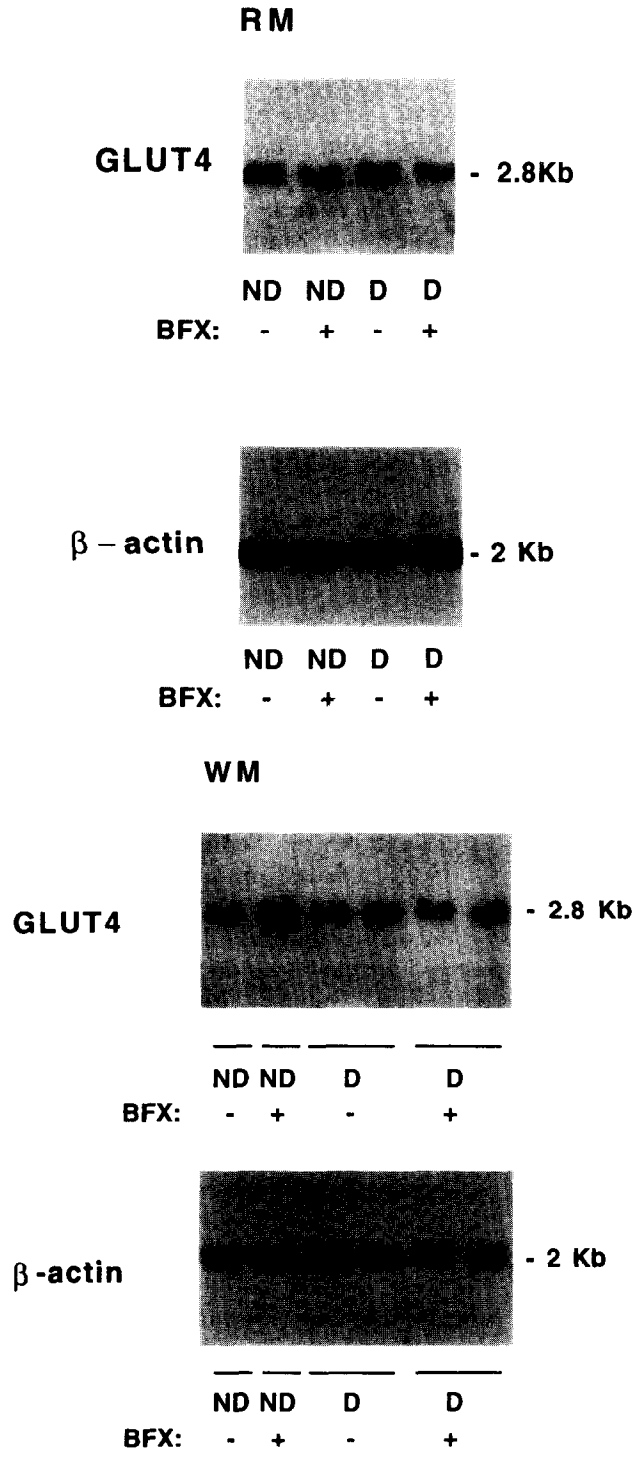

FIG. 4. Effect of diabetes and benfluorex on GLUT4 mRNA levels in muscle. Rats were injected with streptozotocin at day -3 of the experimental protocol. On day 0, benfluorex treatment (35 mg/kg b.w.) was initiated. Total RNA were purified from red (RM, top) and white portions (WM, bottom) of skeletal muscle obtained from untreated (-) or benfluorex-treated (+) nondiabetic (ND) or diabetic (D) rats; 15 μg of total RNA from red and white muscle was applied on gels for Northern blots. GLUT4 mRNA was detected after hybridization with a 2007-base-pair SalI fragment as a cDNA probe. The DNA β-actin probe was a 4500-base-pair HindIII and EcoRI fragment. Representative autoradiograms obtained after different times of exposure are shown.

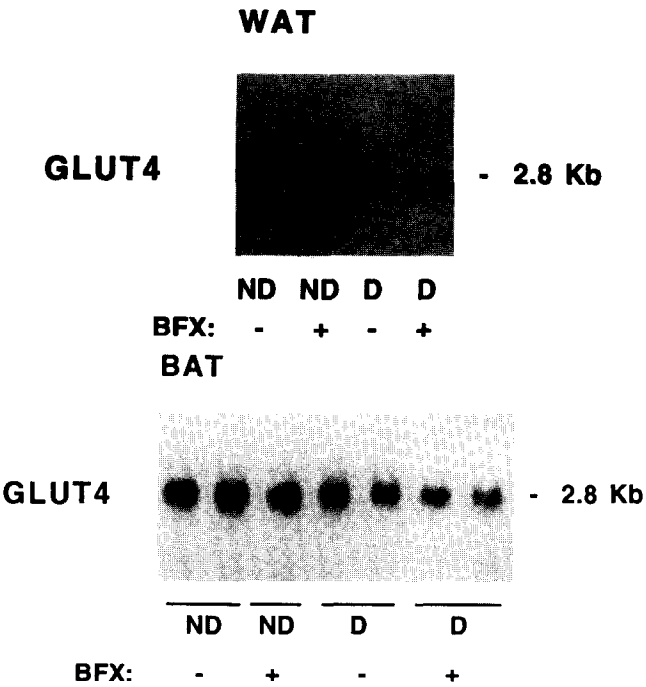

FIG. 5. Effect of diabetes and benfluorex on GLUT4 mRNA levels in adipose tissues. Rats were injected with streptozotocin at day -3 of the experimental protocol. On day 0, benfluorex treatment (35 mg/kg b.w.) was initiated. Total RNA were purified from white (WAT, top) and brown adipose tissues (BAT, bottom) obtained from untreated (-) or benfluorex-treated (+) nondiabetic (ND) or diabetic (D) rats; 15 μg of total RNA from white and brown adipose tissues was applied on gels for Northern blots. GLUT4 mRNA was detected after hybridization with a 2007-base-pair SalI fragment as a cDNA probe. Representative autoradiograms obtained after different times of exposure are shown.

the absence of alterations in GLUT4 mRNA concentrations, (b) chronic benfluorex treatment in normal rats decreased GLUT4 protein but not GLUT4 mRNA in skeletal and cardiac muscle and in brown adipose tissue and (c) benfluorex treatment in diabetic rats led to higher GLUT4 protein levels in brown and white adipose tissues than did treatment in untreated diabetic rats, which occurred in the presence of similar low levels of GLUT4 mRNA. Based on the fact that the membrane protein yield was not modified in the tissues subjected to study in response to benfluorex treatment or streptozotocin-induced diabetes, we think that it is unlikely that these results are due to a different GLUT4 yield. Therefore, we conclude that the changes detected in tissue GLUT4 protein content were the consequence of true alterations in GLUT4 expression. Furthermore, our results indicate that GLUT4 expression in muscle and adipose tissue can be modified by alterations lying at posttranscriptional steps.

The precise mechanisms responsible for the reduction in GLUT4 protein content in muscle and adipose tissue from nondiabetic animals in response to chronic benfluorex treatment are unknown. Based on the fact that chronic malnutrition in the rat does not alter the total content of

GLUT4 in muscle and adipose tissue,‡ we favor the view that benfluorex or its metabolites regulate GLUT4 by acting directly in muscles and brown adipose tissue. However, these data should be interpreted with caution because it is difficult to compare chronic malnutrition, in which animals are subjected to lower amounts of food, with a pharmacologically induced reduction of food intake, as is the case for benfluorex administration.

The results of this and previous studies [9, 16, 17, 39–41] suggest that the regulation of GLUT4 expression by translational or posttranslational steps is a relatively frequent process and can involve either the preservation of GLUT4 carriers in the presence of variable changes in GLUT4 gene expression or the modification of GLUT4 carriers in the absence of changes in GLUT4 mRNA. These two inverse patterns can be explained by changes in the translational efficiency of GLUT4 transcripts in the general machinery of protein synthesis or modifications in the rate of GLUT4 protein degradation. Although there is some information regarding the mechanisms that alter GLUT4 gene transcription in adipose cells and to some extent in muscle cells [42–45], there is an absolute lack of knowledge regarding the specific regulatory mechanisms lying at translational and/or posttranslational level. Much effort should be devoted to unraveling the molecular basis for the control of GLUT4 expression at posttranscriptional steps.

Previously published data suggest that the regulatory mechanisms of GLUT4 expression are different in red and white fibers of skeletal muscle. This suggestion is mainly based on two observations: (a) there is a greater expression of GLUT4 protein in red muscle than in white muscle [9, 46, 47], and (b) fasting and diabetes lead to alterations in the pattern of GLUT4 expression that are clearly different in red and in white muscle [9, 15]. Results obtained in our study further indicate the existence of differential mechanisms involved in the regulation of GLUT4 expression based on the fact that red and white skeletal muscle displayed a differential pattern of response to the combined action of diabetes and benfluorex. Thus, benfluorex treatment for 2 weeks prevented the decrease in total GLUT4 protein content in white muscle associated with diabetes. In contrast, benfluorex and diabetes showed an additive effect by reducing GLUT4 expression in red muscle. The study of the mechanisms involved in the regulation of GLUT4 in red and white muscle requires further work.

Benfluorex treatment has been reported to reduce hyperglycemia in type II diabetes mellitus and increase insulin sensitivity in obese type II diabetic patients [21]. Chronic benfluorex administration also reduces hyperglycemia and reverses hepatic insulin resistance in a rat model of NIDDM induced by injection of a low dose of streptozotocin 5 days after birth [26]. This model of type II diabetes is characterized by hyperglycemia, moderate hypoinsulinemia (50% of normal values) and glucose intolerance [48]. Furthermore, benfluorex treatment prevents the development of insulin resistance in skeletal muscle induced by fat or fructose feeding [28]. To evaluate whether benfluorex also displays antihyperglycemic effects in type I diabetic models, we studied the metabolic impact of chronic benfluorex in streptozotocin-induced diabetes in rats. Our data indicate that benfluorex causes a substantial reduction in food and water intake and in body weight gain, in agreement with previous reports [29, 49]. However, we found no antihyperglycemic effect of benfluorex in this animal model. This result indicates that benfluorex has no antidiabetic action in type I diabetic models and suggests that the anorectic effect of benfluorex is not sufficient for the improvement of glucose metabolism in the diabetic condition.

Our data suggest the necessity of insulin for benfluorex to act as a fully competent antidiabetic agent. Given that benfluorex decreases plasma insulin levels in conditions characterized by hyperinsulinemia such as that after fat feeding, genetic obesity or aging [27–29], some of the beneficial effects of benfluorex treatment might in some way be linked to the restoration of normal insulin concentrations.

Streptozotocin-induced diabetes leads to severe hyperglycemia and is characterized by peripheral insulin resistance and depressed levels of GLUT4 protein in brown adipose tissue, white adipose tissue, skeletal muscle and heart [5–13]. Our data reveal that benfluorex treatment for 2 weeks prevented the decrease in total GLUT4 protein content in white muscle, white adipose tissue and brown adipose tissue associated with diabetes. Based on the lack of alterations in circulating glucose and insulin in benfluorex-treated rats, we conclude that these factors are not responsible for the effects of benfluorex on GLUT4 expression. The benfluorex-induced prevention of GLUT4 protein content in adipose tissues and muscle from diabetic rats did not have a physiological impact on glucose homeostasis in diabetic rats probably because of the lack of sufficient circulating insulin to stimulate the translocation of GLUT4 to the cell surface in muscle and adipose cells. Although we did not study the subcellular distribution of GLUT4 in muscle fibers or adipocytes, most GLUT4 carriers probably are in an intracellular location and therefore inactive both in diabetic and in benfluorex-treated diabetic rats. In this regard, it will be interesting to determine next whether benfluorex treatment also prevents GLUT4 repression associated with type II diabetic states.

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References

1. Gould GW and Holman GD, The glucose transporter family: Structure, function and tissue-specific expression. *Biochem J* **295:** 329–351, 1993.
2. Mueckler M, Facilitative glucose transporters. *Eur J Biochem* **219:** 713–725, 1994.
3. Zorzano A, Wilkinson W, Kotliar N, Thoidis G, Wadzinkski BE, Ruoho AE and Pilch PF, Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J Biol Chem* **264:** 12358–12363, 1989.
4. Marette A, Richardson JM, Ramlal T, Balon TW, Vranic M, Pessin JE and Klip A, Abundance, localization, and insulin-induced translocation of glucose transporters in red and white muscle. *Am J Physiol* **263:** C443–C452, 1992.
5. Berger J, Biswas C, Vicario PP, Strout HV, Saperstein R and Pilch PF, Decreased expression of the insulin-responsive glucose transporter in diabetes and fasting. *Nature* **340:** 70–72, 1989.
6. Sivitz WI, DeSautel SL, Kayano T, Bell GI and Pessin JE, Regulation of glucose transporter messenger RNA in insulin-deficient states. *Natures* **340:** 72–74, 1989.
7. Garvey WT, Huecksteadt TP and Birnbaum MJ, Pretranslational suppression of an insulin-responsive glucose transporter in rats with diabetes mellitus. *Science* **245:** 60–63, 1989.
8. Kahn BB, Charron MJ, Lodish HF, Cushman SW and Flier JS, Differential regulation of two glucose transporters in adipose cells from diabetic and insulin-treated diabetic rats. *J Clin Invest* **84:** 404–411, 1989.
9. Camps M, Castelló A, Muñoz P, Monfar M, Testar X, Palacín M and Zorzano A, Effect of diabetes and fasting on GLUT-4 (muscle/fat) glucose-transporter expression in insulin sensitive tissues. Heterogeneous response in heart, red and white muscle. *Biochem J* **282:** 765–772, 1992.
10. Gerrits PM, Olson AL and Pessin JE, Regulation of the Glut4/muscle–fat glucose transporter messenger-RNA in adipose-tissue of insulin-deficient diabetic rats. *J Biol Chem* **268:** 640–644, 1993.
11. Strout HV, Vicario PP, Biswas C, Saperstein R, Brady EJ, Pilch PF and Berger J, Vanadate treatment of streptozotocin diabetic rats restores expression of the insulin-responsive glucose transporter in skeletal muscle. *Endocrinology* **126:** 2728–2732, 1990.
12. Bourey RE, Koranyi L, James DE, Mueckler M and Permutt MA, Effects of altered glucose homeostasis on glucose transporter expression in skeletal muscle of the rat. *J Clin Invest* **86:** 542–547, 1990.
13. Kahn BB, Rosetti L, Lodish HF and Charron MJ, Decreased in vivo glucose uptake but normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats. *J Clin Invest* **87:** 2197–2206, 1991.
14. Neufer PD, Carey JO and Dohm GL, Transcriptional regulation of the gene for glucose-transporter Glut4 in skeletal-muscle—Effects of diabetes and fasting. *J Biol Chem* **268:** 13824–13829, 1993.
15. Richardson JM, Balon TW, Treadway JL and Pessin JE, Differential regulation of glucose transporter activity and expression in red and white skeletal muscle. *J Biol Chem* **266:** 12690–12694, 1991.
16. Hager SR, Pastorek D, Jochen AL and Meier D, Divergence between GLUT4 mRNA and protein abundance in skeletal muscle of insulin resistant rats. *Biochem Biophys Res Commun* **181:** 240–245, 1991.
17. Castelló A, Rodríguez-Manzaneque JC, Camps M, Pérez-Castillo A, Testar X, Palacín M, Santos A and Zorzano A, Perinatal hypothyroidism impairs the normal transition of GLUT4 and GLUT1 glucose transporters from fetal to new netal levels in heart and brown adipose tissue. Evidence for tissue-specific regulation of GLUT4 expression by thyroid hormone. *J Biol Chem* **269:** 5905–5912, 1994.
18. Arnaud O and Nathan C, Antiobesity and lipid-lowering agents with antidiabetic activity. In: *New Antidiabetic Drugs*. (Eds. Bailey CJ and Flatt PR), pp. 133–142. Smith-Gordon/Nishimura, London, 1990.
19. Vigili de Kreutzenberg S, Riccio A, Dorella M, Da Tos V and Del Prato S, Me!canismes de l'action hypoglycémiante du benfluorex. *La Presse Méd* **21:** 1336–1343, 1992.
20. De Feo P, Lavielle R, De Gregoris P and Bolli GB, Anti-hyperglycemic mechanisms of benfluorex in type II diabetes mellitus. *Diab Metab Rev* **9:** 35S–41S, 1993.
21. Bianchi R, Bongers V, Bravenboer B and Erkelens DW, Effects of benfluorex on insulin resistance and lipid metabolism in obese type II diabetic patients. *Diab Care* **16:** 557–559, 1993.
22. Brindley DN and Bowley M, Effect of fenfluramine and related compounds on the synthesis of glycerolipids by rat liver. *Postgrad Med J* **51:** 91–95, 1975.
23. Duhault J, Boulanger M and Beregi L, Pharmacological effects of 780 SE on hyperlipidaemic and obese rats. *Postgrad Med J* **51:** 99–104, 1975.
24. Marquie G, Effects of 780 SE on lipid metabolism in rabbit aorta. *Eur J Pharmacol* **45:** 1–6, 1977.
25. Geelen MJH, Mechanisms responsible for the inhibitory effects of benfluorex on hepatic intermediary metabolism. *Biochem Pharmacol* **32:** 1765–1772, 1983.
26. Serradas P, Blondel O, Bailbe D and Portha B, Benfluorex normalizes hyperglycemia and reverses hepatic insulin resistance in STZ-induced diabetic rats. *Diabetes* **42:** 564–570, 1993.
27. Duhault J, Boulanger M, Beregi L, Sicot N and Bouvier F, 780 SE: A new type of hypolipidemic agent. Comparative assays in rats. *Atherosclerosis* **23:** 63–72, 1976.
28. Storlien LH, Oakes ND, Pan DA, Kusunoki M and Jenkins AB, Syndromes of insulin resistance in the rat. Inducement by diet and amelioration with benfluorex. *Diabetes* **42:** 457–462, 1993.
29. Brindley DN, Hales P, Al-Sieni AII and Russell JC, Decreased serum lipids, serum insulin and triacylglycerol synthesis in adipose tissue of JCR:LA-corpulent rats treated with benfluorex. *Biochim Biophys Acta* **1085:** 119–125, 1991.
30. Ariano MA, Armstrong RB and Edgerton VR, Hindlimb muscle fiber populations of five mammals. *J Histochem Cytochem* **21:** 51–55, 1973.
31. Baldwin KM, Klinkerfuss GH, Terjung RL, Mole PA and Holloszy JO, Respiratory capacity of white, red and intermediate muscle: adaptive response to exercise. *Am J Physiol* **222:** 373–378, 1972.
32. Goodman MN, Berger M and Ruderman NB, Glucose metabolism in rat skeletal muscle at rest. Effects of starvation, diabetes, ketone bodies, and free fatty acids. *Diabetes* **23:** 881–888, 1974.
33. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
34. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
35. Muñoz P, Rosemblatt M, Testar X, Palacín M and Zorzano A, Isolation and characterization of distinct domains of sarcolemma and T-tubules from rat skeletal muscle. *Biochem J* **307:** 273–280, 1995.
36. Chomczynski P and Sacchi N, Single-step method of RNA

isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
37. Rosen KM and Villa-Komaroff L, An alternative method for the visualization of RNA in formaldehyde agarose gels. *Focus* **12:** 23–24, 1990.
38. Nudel U, Levy Z, Neuman S, Shani M, Yaffe D and Zakut R, The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucleic Acid Res* **11:** 1759–1771, 1983.
39. Santalucía T, Camps M, Castelló A, Muñoz P, Nuel A, Testar X, Palacín M and Zorzano A. Developmental regulation of GLUT-1 (erythroid/hepG2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue. *Endocrinology* **130:** 837–846, 1992.
40. Olichon-Berthe C, Gautier N, Van Obberghen E and Le Marchand-Brustel Y, Expression of the glucose transporter GLUT4 in the muscular dystrophic mdx mouse. *Biochem J* **291:** 257–261, 1993.
41. Pedersen O, Kahn CR and Kahn BB, Divergent regulation of the Glut-1 and Glut-4 glucose transporters in isolated adipocytes from zucker rats. *J Clin Invest* **89:** 1964–1973, 1992.
42. Cornelius P, Pekala PH, Marlowe M and Lee MD, Monokine regulation of glucose transporter messenger-RNA in L6 myotubes. *Biochem Biophys Res Commun* **165:** 429–436, 1989.
43. Kaestner KH, Christy RJ and Lane MD, Mouse insulin-responsive glucose transporter gene characterization of the gene and transactivation by the CAAT enhancer binding protein. *Proc Natl Acad Sci USA* **87:** 251–255, 1990.
44. Stephens JM and Pekala PH, Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. *J Biol Chem* **266:** 21839–21845, 1991.
45. Kaestner KH, Flores-Riveros JR, Mclenithan JC, Janicot M and Lane MD, Transcriptional repression of the mouse insulin-responsive glucose transporter (Glut4) gene by cAMP. *Proc Natl Acad Sci USA* **88:** 1933–1937, 1991.
46. Kern M, Wells JA, Stephens JM, Elton CW, Friedman JE, Tapscott EB, Pekala PH and Dohm GL, Insulin responsiveness in skeletal-muscle is determined by glucose transporter (Glut4) protein level. *Biochem J* **270:** 397–400, 1990.
47. Henriksen EJ, Bourey RE, Rodnick KJ, Koranyi L, Permutt MA and Holloszy JO, Glucose transporter protein content and glucose transport capacity in rat skeletal-muscles. *Am J Physiol* **259:** E593–E598, 1990.
48. Blondel O, Bailbé D and Portha B, Relation of insulin deficiency to impaired insulin action in NIDDM adult rats given streptozotocin as neonates. *Diabetes* **38:** 610–617, 1989.
49. Lacour F, Espinal J, Arnaud O and Duhault J, Improvement in glucose tolerance of insulin resistant rats after chronic or acute administration of benfluorex. *Life Sci* **53:** 1525–1529, 1993.