Regulation of the GLUT4/Muscle-fat Glucose Transporter mRNA in Adipose Tissue of Insulin-deficient Diabetic Rats*

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In contrast to the rapid insulin-dependent translocation of GLUT4, insulin-deficient states such as fasting and streptozotocin (STZ)-induced diabetes results in a specific reduction of GLUT4 mRNA and protein in adipose tissue (11–15). Insulin treatment of STZ-diabetic rats or refeeding of fasted animals stimulates a recovery of both GLUT4 mRNA and protein levels. Under these conditions, the decrease in GLUT4 expression is a major factor contributing to the marked insulin resistance observed in isolated adipocytes (12, 13, 15, 16).

Although these studies indicate that the expression of adipose tissue GLUT4 is under hormonal/metabolic control, the specific in vivo factors responsible have not yet been identified. Recent studies have demonstrated that the regulation of adipose tissue GLUT4 mRNA levels occurs independent of plasma glucose concentration and more closely correlates with changes in insulin levels (16, 17). In contrast, in vitro studies using the cultured 3T3-L1 adipocyte cell line have suggested that GLUT4 mRNA expression is directly regulated by intracellular cAMP levels (18). In this study, we have examined the relationship between adipose tissue cAMP levels and GLUT4 expression in vivo. Furthermore, the expression of GLUT4 in primary isolated rat adipocytes was also assessed.

EXPERIMENTAL PROCEDURES

Animal Model of Insulin-dependent Diabetes—Male Sprague-Dawley rats (160–180 g) were given a single intraperitoneal injection of (125 mg/kg body weight) STZ. Two to three days after injection, plasma glucose concentration was estimated from a tail vein blood sample. Animals were considered diabetic if the glucose levels were greater than 400 mg/dl. The STZ-diabetic animals were either left untreated or given a combination of 2 units of regular insulin (Humulin R) and 3 units of long-acting insulin (Humulin N) per day. Following correction of hyperglycemia, these animals were divided into two groups. One group received subcutaneous injection of 0.3 μmol/kg of phenylisopropyladenosine (PIA) every 2 h or injection with vehicle alone. The animals were killed by CO2 asphyxiation and the adipose tissue was removed for analysis. All procedures were reviewed and approved by The University of Iowa Committee for the Care and Use of Animals. Untreated, nondiabetic animals of similar age and weight were used as controls.

Isolation of Primary Rat Adipocytes—Adipocytes were isolated from whole epididymal fat pads of 150–200-g Sprague-Dawley male rats by collagenase digestion as described by Rodbell (19). The fat pads were digested for 1 h at 37 °C in Krebs-Ringer-Hepes buffer, pH 7.4, containing 1% bovine serum albumin and 1.0 mg/ml collagenase (Clostridium histolyticum, Sigma). The isolated fat cells were washed twice using the above buffer without collagenase and the cells were incubated for various times at 37 °C in 10 ml of Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum as described by Marshall et al. (20).

Determination of cAMP Levels—Adipose tissue and isolated adipocytes were snap frozen in liquid nitrogen and stored at −70 °C until analyzed. cAMP concentration was measured using a RAININ cAMP [3H]labeled radioimmunoassay kit (Amersham Corp.). The assay was

Facilitative glucose transport in mammalian cells is mediated by a family of structurally related glucose transporter proteins (1–3). Although most tissues express more than one glucose transporter isoform, GLUT4 is the major glucose transporter expressed in adipose, skeletal, and cardiac muscle. In these tissues, acute insulin stimulation results in the predominant translocation of an intracellular pool of preformed GLUT4 glucose transporters to the plasma membrane (4–10).

The increase in cell surface GLUT4 protein directly correlates with the increase in glucose transport activity and accounts for most, if not all, of the stimulation of glucose uptake by insulin (7–10).

Previous studies have documented that streptozotocin-induced insulin deficiency results in a marked decrease in adipose tissue GLUT4 glucose transporter mRNA levels (Sivitz, W. I., DeSautel, S. L., Kayano, T., Bell, G. I., and Pessin, J. E. (1989) Nature 340, 72–74). In this study, nuclear run-on analysis performed on diabetic and insulin-treated diabetic rats demonstrated that the decrease in GLUT4 mRNA occurs via a diabetes-induced decrease in GLUT4 transcription rate. The decrease in GLUT4 mRNA levels could be prevented by treatment of the diabetic animals with the adenosine receptor agonist phenylisopropyladenosine (PIA). Under these conditions, PIA completely blocked the elevation of intracellular cAMP levels associated with insulin deficiency.

Surprisingly, isolation of primary rat adipocytes from control animals resulted in a rapid decrease (~20-fold) in GLUT4 mRNA levels by 24 h with a concomitant increase (~70-fold) in GLUT1 mRNA levels. This rapid loss of GLUT4 expression did not correlate with changes in adipocyte cAMP levels and was not prevented by treatment of the cells with either insulin and/or PIA. These data demonstrate that the decrease in GLUT4 transcription induced by insulin deficiency in vitro predominantly results from an increase in intracellular cAMP levels. In contrast, although GLUT4 transcription also decreases in adipocytes when removed from their normal physiological environment, this occurs independent of changes in cAMP levels.

In vivo.

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The abbreviations used are: STZ, streptozotocin; cAMP, 3′,5′-cyclic adenosine monophosphate; PIA, phenylisopropyladenosine.

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done using extracts obtained by homogenization in 6% trichloroacetic acid followed by extraction with water-saturated ether. Results were expressed as picomole/mg for the tissue samples or picomole/10^6 cells for isolated primary adipocytes.

**Nuclei Isolation—**GLUT4 transcription rate was measured in adipose tissue from STZ-diabetic and insulin-treated STZ-diabetic animals. Nuclei were isolated from adipose tissue by homogenization using a Dounce homogenizer with a loose-fitting pestle in Buffer I (60 mM KCl, 15 mM NaCl, 15 mM Hepes, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and 14 mM 2-mercaptoethanol) containing 0.3 M sucrose. The homogenate was filtered with sterile gauze, overlayed on an equal volume of Buffer II (60 mM KCl, 15 mM NaCl, 15 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and 14 mM 2-mercaptoethanol) containing 2.0 M sucrose and homogenized in a Dounce homogenizer with a tight-fitting pestle. The homogenate was overlayed on 3 ml of Buffer II and centrifuged at 30,000 rpm for 65 min in a SW-41 rotor (Beckman) at 4 °C. The pellet was resuspended in 0.5 ml of nuclei storage buffer (50% glycerol, 50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 10 mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride) and repelleted by microcentrifugation for 2.5 min. The final pellet was resuspended in 0.05 ml of nuclei storage buffer and frozen at -70 °C.

**Nuclear Run-on Transcription—**Transcription assays were carried out in a 0.1-ml reaction volume in buffer containing 32% glycerol, 100 mM KCl, 50 mM Hepes, pH 7.4, 5 mM magnesium acetate, 4 mM dithiothreitol, 0.1 mM EDTA, 4 units of RNasin, 1 mM each CTP, GTP, and ATP plus 0.5 ml of [32P]UTP. The reaction was allowed to proceed for 15 min at 28 °C and terminated by the addition of 1 ml MgCl₂ and 7000 units/ml RNase-free DNase I for 15 min at 37 °C. The reaction mixture was then digested with 300 μg/ml proteinase K in the presence of 0.1% SDS. The labeled RNA transcripts were isolated using the acid/phenol method (21) and resuspended in 100 μl of water.

Nylon filters (Nytran, Schleicher and Schuell, Inc.) containing fixed and denatured cDNAs (5 μg) were prehybridized for 1 h at 50 °C in 6 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaPO₄, pH 7.7, 1 mM EDTA), 10 × Denhardt’s reagent, 1.0% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at 65 °C for 48 h in 50% deionized formamide, 6 × SSPE, 1.0% SDS, and 100 μg/ml denatured salmon sperm DNA plus 7 × 10⁶ cpm/ml of radiolabeled RNA transcripts. Subsequent to hybridization, the filters were washed 3 times under low stringency conditions (1 × SSPE, 1.0% SDS) at 65 °C and a single high stringency wash (0.1 × SSPE, 1.0% SDS) at 65 °C followed by treatment with ribonuclease A (10 μg/ml in 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at 37 °C. The washed filters were subjected to autoradiography and quantitated by laser scanning densitometry (LKB Urascan).

**RNA Isolation—**Adipose tissue or primary isolated rat adipocytes were snap frozen in liquid nitrogen and total cellular RNA was obtained by acid/phenol extraction (21). RNA was quantitated spectrophotometrically by absorbance at 260 nm and stored as an ethanol precipitate at -70 °C.

**Northern Blot Analysis—**Aliquots of 10 μg of total RNA were fractionated by 1% agarose-formaldehyde gel electrophoresis. After electrophoresis, RNA was transferred to Nytran filters (0.45 μm) and the filters were prehybridized for 1 h at 60 °C in a solution of 50% deionized formamide, 5 × Denhardt’s reagent, 1.0% SDS, and 200 μg/ml denatured salmon sperm DNA. Hybridization of filters was carried out with fresh prehybridization buffer solution containing 2 × 10⁶ cpm/ml of radiolabeled probe. The probe was an antisense RNA derived from rat GLUT4 cDNA (pS1-1) that was linearized with EcoRV and transcribed using T7 polymerase (22). Hybridization was carried out overnight at 60 °C. Filters were washed according to the manufacturer’s specifications which included a high stringency wash with RNase A (10 μg/ml in 2 × SSPE) at 37 °C for 15 min. The washed filters were exposed to Kodak XAR film at -70 °C.

**RESULTS**

Previous studies have documented a substantial decrease in rat adipose tissue steady-state GLUT4 mRNA levels in insulin-deficient states (11, 13-15). These variations in GLUT4 mRNA levels could result from alterations in either GLUT4 mRNA synthesis and/or degradation. To examine the effect of STZ-diabetes on GLUT4 mRNA synthesis, nuclear run-on assays were performed (Fig. 1). Transcriptionally active nuclei were isolated from adipose tissue of STZ-diabetic (Fig. 1, lane 1) and insulin-treated STZ-diabetic (Fig. 1, lane 2) rats. The adipose tissue GLUT4 transcription rate in diabetic rats was substantially lower (~10-fold) compared to nuclei isolated from insulin-treated STZ-diabetic animals. As internal controls, the in vitro transcription rate of total RNA (genomic) and β-actin RNA were unchanged under these conditions. Consistent with the low levels of adipose tissue GLUT1 mRNA, the transcription rate of GLUT1 was extremely low in these assays (data not shown). These data directly demonstrate that the major effect of STZ-induced insulin deficiency was to decrease GLUT4 transcription in adipose tissue which was subsequently increased by insulin therapy.

Insulin deficiency is associated with a marked increase in adipose cell catabolism (i.e. lipolysis) due to elevation of intracellular cAMP levels. Since previous studies have demonstrated that adenosine receptor agonists can inhibit insulin-deficient diabetes-induced lipolysis in vivo (23), we examined the effect of PIA on adipose cell cAMP levels (Fig. 2). Steady-state intracellular cAMP levels in adipose tissue was approximately 0.2 pmol/mg protein in control animals and were elevated to approximately 0.5 pmol/mg following 3 days of STZ-induced diabetes. As expected, insulin treatment of the STZ-diabetic animals resulted in the normalization of adipose tissue cAMP levels back towards control values (Fig. 2A). To determine if PIA treatment could prevent the elevation of cAMP levels, STZ-diabetic animals were treated with insulin overnight, followed by repetitive 2-h PIA or saline injections for the following 10 h. Under these conditions, PIA completely inhibited the elevation of adipose cAMP levels which occurred in the saline-treated animals (Fig. 2B).

In parallel to cAMP determination, RNA was isolated from the contralateral epididymal fat pads and analyzed by Northern blotting for GLUT4 and β-actin mRNA (Fig. 3). Consistent with previous studies (11, 13-15), STZ-diabetes resulted in a specific decrease in adipose tissue steady-state GLUT4 mRNA levels (Fig. 3A, lane 2) compared to control animals (Fig. 3A, lane 1) and which fully recovered following insulin treatment (Fig. 3A, lane 3). Replacement of insulin therapy by saline injections resulted in the expected loss of GLUT4 mRNA levels (Fig. 3A, lane 5). However, initiation of PIA treatment completely prevented the decrease in adipose tissue GLUT4 mRNA levels that occurs in the absence of insulin (Fig. 3A, lane 4). As controls, there was no significant change in the relative levels of β-actin mRNA under these conditions (Fig. 3B, lanes 1-5).

To further assess the relationship between adipose tissue

![FIG. 1. Nuclear run-on transcription assay using nuclei from STZ-diabetic and insulin-treated diabetic rat epididymal fat pads. A standard nuclear run-on transcription assay was performed and the labeled RNA transcripts were hybridized to full-length cDNAs for GLUT4, β-actin, vector, and genomic DNA which were immobilized on nylon filters as described under "Experimental Procedures." Lane 1 is from 2-day STZ-diabetic (D) and lane 2 is from 2-day STZ-diabetic animals treated with insulin for 5 h (D+I).](image-url)
Asterisk, were determined in adipose tissue from untreated either saline by radioimmunoassay as described under "Experimental Procedures." The results shown are the mean ± S.E. from three experiments. There were no significant differences between control and insulin-treated diabetic animals.

![Graph showing cAMP levels in epididymal fat pads](image)

**Fig. 2.** cAMP levels in epididymal fat pads. A, cAMP levels were determined in adipose tissue from untreated (control), 3-day STZ-diabetic (diabetic), and overnight insulin-treated STZ-diabetic rats (insulin). B, the STZ-diabetic rats which were treated with insulin overnight were subjected to an additional 10-h treatment with either saline (saline) or PIA (PIA) injections. cAMP was measured by radioimmunoassay as described under "Experimental Procedures." The results shown are the mean ± S.E. from three experiments. There were no significant differences between control and insulin-treated diabetic animals.

![Northern blot analysis](image)

**Fig. 3.** Northern blot analysis of RNA isolated from epididymal rat fat pads. Total RNA was isolated from control (lane 1), 3-day STZ-diabetic (lane 2), and overnight insulin-treated STZ-diabetic rats (lane 3). The STZ-diabetic rats which were treated with insulin overnight were then subjected to an additional 10-h treatment with either saline (saline) or PIA (PIA) injections. Ten μg of total cellular RNA was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to nylon filters, and probed with 32P-labeled antisense RNA. A, total cellular RNA probed for GLUT4 mRNA. B, total cellular RNA probed for β-actin mRNA.

CAMP and GLUT4 mRNA levels, we examined the time-dependent effect of insulin withdrawal in the absence and presence of PIA (Fig. 4). STZ-induced diabetes resulted in the expected decrease in steady-state GLUT4 mRNA levels (Fig. 4A, lane 2) compared to control animals (Fig. 4A, lane 1) and which again recovered following insulin therapy (Fig. 4A, lane 3). Termination of insulin therapy resulted in a rapid loss of GLUT4 mRNA (Fig. 4A, lanes 5, 7, 9, 11, and 13) which was first detected by 6 h (Fig. 4A, compare lane 3 with lane 7) and was maximal by 10–12 h (Fig. 4A, compare lane 3 with lanes 11 and 13). In contrast, replacement of insulin therapy with PIA injections completely prevented this decrease in adipose tissue GLUT4 mRNA levels (Fig. 4A, lanes 4, 6, 8, 10, and 12). Under both of these conditions, the steady-state expression levels of β-actin was not significantly changed during this time period (Fig. 4B, lanes 1–13).

Examination of the time-dependent changes in cAMP levels upon termination of insulin treatment demonstrated a marked increase in cAMP content between 6 and 8 h following cessation of insulin therapy (Fig. 5). Quantitation of the changes in GLUT4 mRNA by scanning laser densitometry demonstrated a inverse relationship between GLUT4 mRNA levels and cAMP accumulation with a t½ of approximately 6 and 7 h, respectively. The effect of PIA to completely block cAMP accumulation directly paralleled the preservation of GLUT4 mRNA under these conditions.

To further examine this relationship, we next isolated pri-
mRNA was found to rapidly decrease upon cell isolation (Fig. 6A). The relative level of GLUT4 mRNA was slightly decreased following the 1-h collagenase digestion, which was required for the isolation of adipocytes (Fig. 6A, lane 2) compared to adipose tissue (Fig. 6A, lane 1). Incubation of the isolated adipocytes under standard tissue culture conditions (20) resulted in a progressive decrease in GLUT4 mRNA levels which were reduced approximately 20-fold within 24 h (Fig. 6A, lanes 3–6). The progressive decline in GLUT4 expression was not due to cell death as there was no significant change in β-actin mRNA in the same samples (data not shown). In contrast, GLUT1 mRNA was barely detectable in adipose tissue (Fig. 6B, lane 1) or immediately following adipocyte isolation (Fig. 6B, lane 2). However, GLUT1 mRNA levels rapidly increased as a function of cell culture time in direct opposition to the decrease in GLUT4 mRNA levels (Fig. 6B, lanes 3–6). Quantitation of these data by laser scanning densitometry indicated that the decrease in GLUT4 mRNA levels slightly preceded the increase in GLUT1 mRNA expression (Fig. 6C).

Since elevated cAMP in vivo was previously observed to decrease GLUT4 mRNA levels (Figs. 3 and 4), we examined the effect of PIA treatment on isolated primary adipocytes (Fig. 7). Normal rats were either left untreated or treated with insulin, PIA, and insulin plus PIA for 4 h prior to isolation of primary adipocytes in media containing these agents. As previously observed, there was a small decrease in GLUT4 mRNA levels immediately following adipocyte cell isolation (Fig. 7, lane 2) compared to intact tissue (Fig. 7, lane 1), whereas GLUT4 mRNA was substantially reduced by 14 h of primary cell culture (Fig. 7, lane 3). Similarly, insulin treatment did not antagonize the loss of GLUT4 mRNA upon adipocyte isolation (Fig. 7, lanes 4–6). Although PIA (Fig. 7, lane 7) and PIA plus insulin (Fig. 7, lane 10) increased the adipose tissue content of GLUT4 mRNA levels, these agents were also unable to prevent the time-dependent decrease in GLUT4 expression which occurred in the primary isolated adipocyte cell cultures (Fig. 7, lanes 9 and 12).

In parallel, intracellular levels of cAMP were determined in the isolated primary adipocytes either untreated or treated with insulin and/or PIA for 14 h (Fig. 8). cAMP levels of untreated primary adipocytes were found not to be significantly different from insulin-treated adipocytes. However, PIA and PIA plus insulin treatments did reduce the cellular cAMP content approximately 50%. Nevertheless, PIA did not inhibit the decrease in GLUT4 mRNA levels (Fig. 7). Thus, in direct contrast to adipose tissue in vivo, the decrease in GLUT4 mRNA upon isolation of primary adipocytes was not related to changes in intracellular cAMP content.

**DISCUSSION**

The mechanism by which peripheral tissue insulin resistance of glucose transport activity occurs is a central issue for...
our understanding of diabetes and obesity. A variety of studies have suggested that insulin resistance in both adipose and muscle tissue can result from an uncoupling of insulin's ability to induce GLUT4 translocation and/or activation (24-26). In addition, several states of insulin resistance have also been observed to directly correlate with a decreased expression of adipose GLUT4 mRNA which results in a down-regulation of GLUT4 protein (11-15). Under these conditions, the intracellular pool of GLUT4 protein becomes depleted such that insulin-stimulated translocation results in a marked reduction in the number of recruitable GLUT4 proteins.

To assess the molecular basis for the STZ-induced decrease in rat adipose tissue steady-state GLUT4 mRNA levels, we initially performed nuclear run-on analysis. These data demonstrate that STZ-diabetes specifically decreases the transcription rate of the GLUT4 gene which accounts for the major, if not all, the decrease in GLUT4 mRNA. The loss of GLUT4 expression is a function of insulin deficiency per se as changes in glycemia do not correlate with the changes in GLUT4 mRNA (16, 17). However, insulin deficiency effects multiple counter-regulatory hormones, particularly those which elevate intracellular cAMP levels in adipocytes (27). In this regard, recent in vitro studies using the differentiating murine 3T3-L1 adipocyte cell line have demonstrated that elevated cAMP decreases GLUT4 expression primarily via an inhibition of transcription (18). Surprisingly, cAMP was also observed to increase GLUT1 expression by both transcriptional and post-transcriptional mechanisms. This is in direct contrast to the in vivo situation where insulin deficiency, either STZ-induced diabetes or fasting, markedly decreases GLUT4 mRNA and protein levels without any significant effect on GLUT1 expression (11-15).

Thus, to determine whether the decreased GLUT4 transcription rate in vivo was a result of elevated adipocyte intracellular cAMP levels, STZ-diabetic animals were treated with an adenosine receptor agonist (PIA) which completely blocked this effect on GLUT1 expression (11-15). Under these conditions, the intracellular pool of GLUT4 protein becomes depleted such that insulin-stimulated translocation results in a marked reduction in the number of recruitable GLUT4 proteins.

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