Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 338-346

TRANSFECTION OF DNA INTO ISOLATED RAT ADIPOSE CELLS BY ELECTROPORATION: Evaluation of promoter activity in transfected adipose cells which are highly responsive to insulin after one day in culture

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Received May 19, 1993

SUMMARY: Isolated adipose cells are among the most insulin responsive cells with respect to glucose transport and metabolism. However, molecular biological techniques such as transfection of DNA have heretofore not been applied successfully in these cells in primary culture. We report a method for transfection of DNA into rat adipose cells by electroporation. Six shocks at 800 V and 25 μF in a 0.4 cm gap cuvette results in efficient transfection. We compared the ability of five promoters to drive expression of a luciferase reporter gene in transfected adipose cells. After one day in culture, promoter activity ranged from no expression to a very high level of expression. These transfected, cultured cells also displayed a 10-fold increase in 3-O-methylglucose transport with maximal insulin stimulation. The ability to transfect DNA into adipose cells which remain insulin responsive after one day in primary culture may be helpful for understanding adipose cell-specific gene regulation and elucidating the molecular mechanisms of insulin action.

Insulin sensitive tissues such as muscle and fat are important in maintaining normal glucose homeostasis (1). Abnormalities in insulin action in these tissues have been implicated in the pathophysiology of diabetes (2). In adipose cells in primary culture, application of molecular biological approaches such as transfection of DNA have not previously been reported. Because adipose cells are technically difficult to work with, tissue culture cell lines have been developed as models. For example, 3T3-L1 and 3T3-F442A cells lines are derivatives of mouse fibroblasts which differentiate into adipocyte-like phenotypes under appropriate conditions (3,4). While these cell lines have been extremely useful, they do not always differentiate completely and uniformly. In addition, even fully differentiated 3T3-L1 cells are not as responsive to insulin as primary adipose cells. Finally, since tissue culture lines have been immortalized, they

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0006-291X/93 $4.00

have characteristics not found in normal cells (e.g., aneuploidy). We report a method for transfection of DNA into isolated rat adipose cells by electroporation. This is the first report of a protocol for transfection of a *bona fide* insulin target cell in primary culture. Transfection conditions were optimized using a luciferase reporter gene driven by an RSV promoter. The activities of five different promoters were compared in transfected adipose cells after one day in culture. Finally, we demonstrate that transfected, cultured adipose cells are highly responsive to insulin as assessed by 3-O-methylglucose transport.

METHODS

Materials - D-[^{14}C]-3-O-Methylglucose (57.1 mCi/mmol), L-[1-^{3}H(N)]-glucose (20 Ci/mmol), and [^{125}I]insulin (receptor grade, 360 Ci/g) were obtained from DuPont NEN (Wilmington, DE). Restriction endonucleases were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). DNA ligase and gel purification kits were obtained from Takara Biochemical Inc. (Berkeley, CA). Sequenase 2.0 was obtained from United States Biochemical Corp. (Cleveland, OH). Chemicals were obtained from Sigma (St. Louis, MO).

DNA vector constructions -

RSV-luc - A plasmid containing a luciferase reporter gene under the control of the Rous sarcoma virus (RSV) long terminal repeat promoter was obtained from Dr. Charles Roberts (5). This plasmid, designated RSV-luc, was the source of the luciferase reporter gene used in the subsequent constructions.

PCIS2-luc - The PCIS2 vector was obtained from Dr. Cornelia Gorman (6). The HindIII site in the HindIII/SmaI fragment from RSV-luc containing the luciferase gene was partially filled in with nucleotides A and G using Sequenase 2.0. The PCIS2 vector was digested with XbaI and HpaI. Using Sequenase 2.0, the XbaI site was partially filled in with nucleotides C and T to generate a complementary end to the partially filled in HindIII site in the DNA fragment derived from RSV-luc. Ligation resulted in a plasmid with the CMV promoter and enhancer with a generic intron in the 5' position driving the luciferase reporter gene.

SRα-luc - The HindIII/SmaI fragment from RSV-luc containing the luciferase reporter gene was ligated into a vector containing the SRα promoter (SV40 early promoter with R-U5 segment of HTLV-I long terminal repeat) (7) obtained from Dr. Richard Roth. The SRα vector had been modified by replacing the PstI/KpnI fragment with the PstI/KpnI fragment from the pUC18 polylinker (8).

SV232A-luc - This vector, obtained from Dr. Thierry Pineau, contains the luciferase gene under the control of the SV40 enhancerless promoter (6).

pcDNAI-luc - The pcDNAI vector containing the CMV promoter and enhancer was purchased from InVitrogen (San Diego, CA). The HindIII/SmaI fragment containing the luciferase reporter gene from RSV-luc was ligated into the HindIII/EcoRV sites in pcDNAI.

pGEM7-luc - The pGEM7zf(+) plasmid was purchased from Promega (Madison, WI). The HindIII/SmaI fragment from RSV-luc containing the luciferase reporter gene was ligated into the HindIII/SmaI site of pGEM7zf(+). This vector served as a negative control since the pGEM7zf(+) plasmid has no eukaryotic promoter.

Milligram quantities of the plasmid DNA vectors described above were obtained using a Magic Maxiprep kit (Promega; Madison, WI). The concentration of plasmid DNA was determined by comparison to known DNA markers using ethidium bromide staining of restriction-digested plasmid run on an agarose gel.

Isolated rat adipose cell preparation - Isolated adipose cells were prepared from the epididymal fat pads of male rats (170-200 g, CD strain, Charles River Breeding Laboratories, Wilmington, MA) by collagenase digestion as previously described (9). The cells were washed

3 times in Krebs-Ringer medium, pH 7.4, containing 10 mM $NaHCO_3$, 30 mM HEPES, 200 nM adenosine, and 1% (w/v) bovine serum albumin. The cells were then washed twice with DMEM, pH 7.4 (containing 2 mM glutamine, 200 nM (R)-N^6-(1-methyl-2-phenylethyl)adenosine (PIA), gentamicin 100 μg/ml, and 25 mM HEPES). The cells were resuspended in this DMEM at a cytocrit of approximately 40%.

Electroporation - 0.2 ml of Dulbecco's PBS with Ca^{++} and Mg^{++} was placed in a 0.4 cm gap electroporation cuvette (Bio-Rad #165-2090; Richmond, CA) along with 0.2 to 20 μg of plasmid DNA. 0.2 ml of a 40% cytocrit preparation of rat adipose cells was added to each cuvette and mixed with the PBS. Electroporation was performed using a Gene Pulser Transfection Apparatus (Bio-Rad; Richmond, CA). Unless otherwise noted, 6 shocks were administered at a voltage of 800 V and a capacitance of 25 μF. The polarity of electroporation was reversed after the first three shocks. The time constant of electroporation was typically 0.6 msec during the final shock. After electroporation, the cells were transferred to 17 mm x 100 mm polystyrene tubes (Falcon 2059) and incubated at 37 °C in 5% CO_2. Two hr after electroporation, 0.5 ml DMEM, pH 7.4 (containing 7% albumin, 2 mM glutamine, 200 nM PIA, gentamicin 100 μg/ml, and 25 mM HEPES) was added to each tube. After 16 to 24 hr of incubation at 37 °C in 5% CO_2, the cells were assayed for luciferase activity and insulin binding.

For the 3-O-methylglucose transport experiments, cells from 15 cuvettes were pooled and placed in 6 cm petri dishes (Falcon 3002) after electroporation. After 2 hr of incubation at 37 °C in 5% CO_2, 6 ml of DMEM with 7% albumin was added to each dish. The cells were assayed for 3-O-methylglucose transport after incubation at 37 °C in 5% CO_2 for 16 to 24 hr.

Luciferase assay - Luciferase activity was assessed using the Promega Luciferase Assay System (Promega; Madison, WI) in conjunction with a Clinilumat luminometer (Berthold Analytical Instruments; Nashua, NH). After electroporation and incubation for 16 to 24 hr, a 0.3 ml aliquot of cells was removed from the incubation tubes and placed in a 1.5 ml Eppendorf tube and the cells separated by settling at unit gravity. The infranatant was removed, and 50 μl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diamincyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added to each sample. After 10 min, the sample was vortexed and 20 μl of sample was mixed with 50 μl of luciferin substrate (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP) in a 12 mm x 75 mm glass test tube (Kimble). Luciferase activity in each sample was assayed immediately after the substrate was added (measurement time of 30 seconds). In some cases, the sample was diluted 1:10 with lysis buffer in order to stay within the linear range of the luminometer.

Adipose cell counting - Aliquots of cells were fixed using osmium tetroxide as previously described (10). The number of cells in each aliquot was counted using an automated Coulter cell counter.

Insulin binding - Insulin binding studies were performed at room temperature (25 °C) on cells which had been electroporated and cultured for 16 to 24 hr. [^{125}I]Insulin was added to binding buffer (NaCl, 120 mM; $MgSO_4$, 1.2 mM; KCl, 2.5 mM; Na acetate, 15 mM; glucose, 10 mM; EDTA, 1mM; HEPES, 50 mM, pH 7.8; bovine serum albumin 10 mg/ml) at tracer concentrations (approximately 0.2 ng/ml). 400 μl of this tracer insulin was added to a 400 μl aliquot of cells. After 45 min, 300 μl aliquots of the binding mixture were placed in polypropylene microcentrifuge tubes (4 mm x 45 mm) containing 100 μl of dinonylphthalate oil. The cells were rapidly separated from the binding buffer by centrifugation at 10,000 x g for 5 seconds. Cell-associated radioactivity was counted in a gamma counter. The percent binding was calculated as the cell-associated counts divided by the total counts added to the incubation. To compare the number of binding sites between samples, the bound/free ratio was calculated.

3-O-methylglucose transport - This assay was performed as described previously (9). For each experiment, a portion of the freshly isolated cells was immediately assayed for insulin-stimulated 3-O-methylglucose transport. The remainder of the cells was subject to transfection

and 16-24 hr of incubation at 37 °C in 5% CO_2 prior to the measurement of 3-O-methylglucose transport at insulin concentrations of 0, 0.07, and 700 nM.

RESULTS

Optimization of transfection protocol in rat adipose cells - We used the RSV-luc and PCIS2-luc vectors to optimize our transfection conditions. Altering voltage, capacitance, buffer volume, DNA concentration, and number of shocks (Table 1) resulted in a range of luciferase activities which varied over three orders of magnitude (Figs. 1 and 2). Sixteen to 24 hr after transfection, the highest activity was observed for conditions of 800 V, 25 μF, 6 shocks, and a mixture of 0.2 ml PBS with 0.2 ml cells (20% final cytocrit). To exclude the possibility of false positive results, we assayed cells which had been incubated with RSV-luc and underwent identical preparation and treatment except for electroporation. In addition, we transfected pGEM7-luc (a promoterless vector) into adipose cells using our optimized protocol. In neither case were we able to detect luciferase activity. Furthermore, luciferase was not detectable in RSV-luc transfected cells which were assayed 3 hr after electroporation (data not shown).

The luciferase activity present in the transfected adipose cells after one day in culture was positively correlated with the number of shocks administered and the amount of DNA used for transfection (Figs. 2a and 2c). However, dose-dependent lethal effects of both DNA and electroporation were observed since the relative numbers of cells (as assessed by insulin binding) decreased as either DNA concentration or number of shocks increased (Figs. 2b and 2d). The number of intact cells counted after osmium tetroxide fixation correlated well with the bound/free ratio calculated for tracer insulin binding in both freshly isolated and electroporated cells (data not shown).

Promoter specific effects in rat adipose cells - Using our optimized transfection protocol, we tested the ability of five different promoters to drive a luciferase reporter gene in isolated rat adipose cells (Fig. 3a). For each vector that generated measurable luciferase activity, the level of expression of luciferase was positively correlated with the amount of vector DNA used for

Table 1. Electroporation conditions used to optimize transfection protocol for isolated rat adipose cells

Condition	Cell Suspension (ml)	PBS (ml)	Voltage (V)	Capacitance (μF)	Shocks (#)
1	0.1	0.3	180	960	1
2	0.2	0.2	180	960	1
3	0.1	0.3	800	25	6
4	0.2	0.2	800	25	6

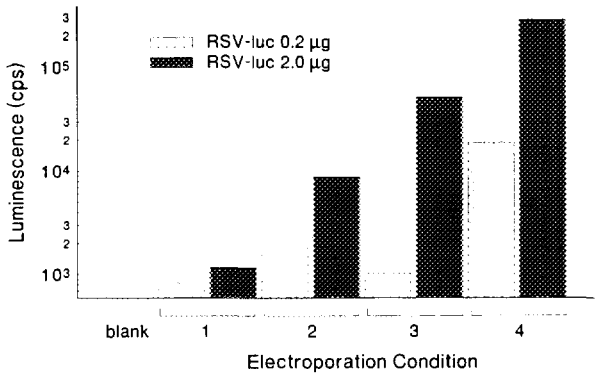

Figure 1. Luciferase activity in rat adipose cells transfected under various electroporation conditions. Table 1 describes the four conditions tested in this experiment. Each condition was tested with 0.2 and 2.0 μg of RSV-luc DNA/cuvette. Luminescence is plotted on the y-axis using a log scale.

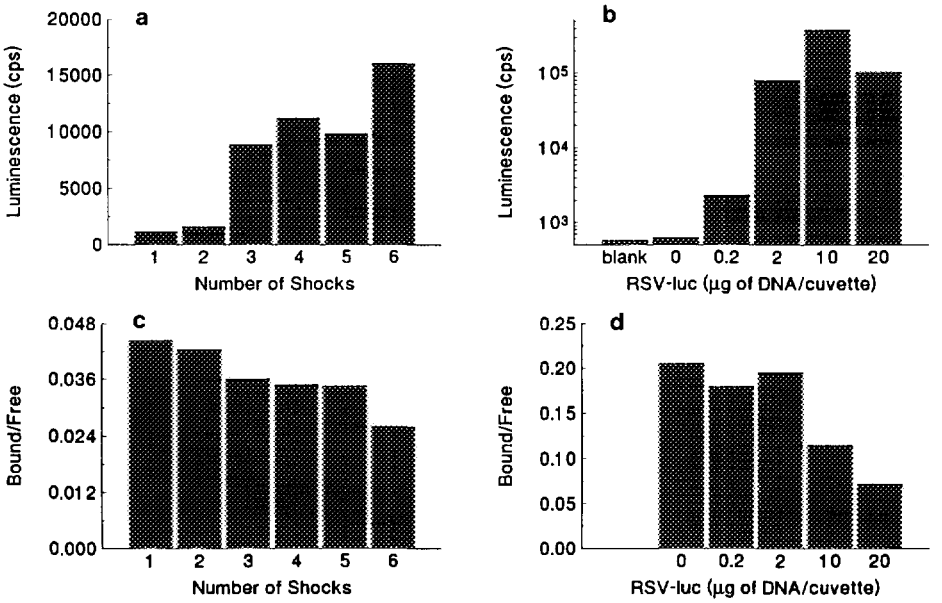

Figure 2a-d. The effects of number of shocks and DNA concentration on luciferase activity and insulin binding. These experiments were repeated twice in duplicate with similar results. **a.** Luciferase activity in rat adipose cells transfected with 0.2 μg PCIS2-luc DNA/cuvette using 800 V, 25 μF and 1-6 shocks (mean of duplicate cuvettes). **b.** Insulin binding to cells from panel **a** (mean of two points assayed in duplicate). The bound/free ratio is proportional to the number of binding sites and the number of cells. **c.** Luciferase activity in cells transfected with 0.2 - 20 μg RSV-luc DNA/cuvette using 6 shocks, 800 V, and 25 μF. Note that the luminescence is plotted on the y-axis using a log scale. **d.** Insulin binding to cells from panel **c** (mean of duplicate samples).

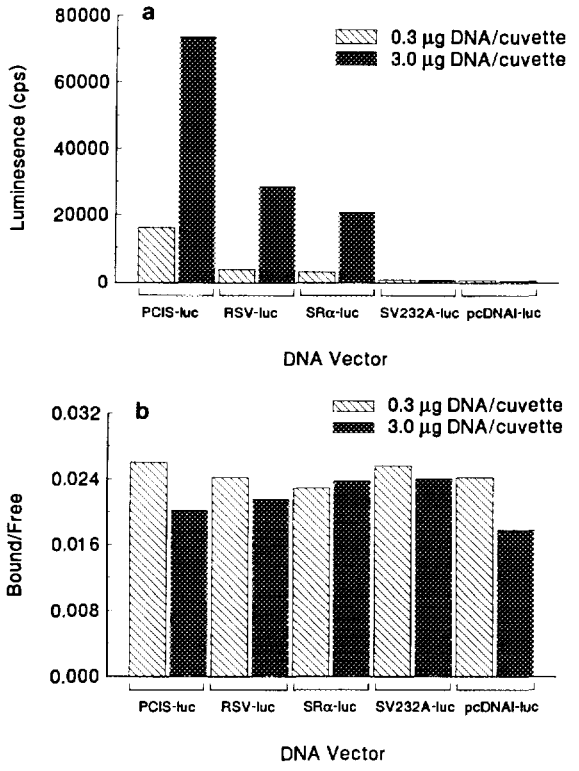

Figure 3ab. Promoter activity in rat adipose cells. These experiments were repeated twice in duplicate with similar results. Each vector was transfected using 0.3 and 3.0 µg of DNA/cuvette. **a.** Luciferase activity in cells transfected with different promoters (mean of duplicate cuvettes). The SV232A-luc activity was approximately 2-3 times the background counts while the pcDNAI-luc activity was indistinguishable from the background counts. **b.** Insulin binding to cells from panel **a** (mean of two points assayed in duplicate).

transfection. The PCIS2-luc vector, containing the CMV promoter/enhancer with a generic intron in the 5' position, generated the highest luciferase activity. RSV-luc and SRα-luc, containing the RSV long terminal repeat promoter and the SV40 promoter with HTLV-I long terminal repeat respectively, generated moderate luciferase activity. SV232A-luc, containing the SV40 promoter without an enhancer, generated weak activity (approximately 2-3 times the background activity). pcDNAI-luc, containing the CMV promoter/enhancer in the absence of an upstream intron, did not generate detectable luciferase activity. The number of viable cells left after transfection and culture (assessed by insulin binding) was comparable for each of the promoters tested (Fig. 3b).

Insulin-stimulated 3-O-methylglucose transport - We measured insulin-stimulated 3-O-methylglucose transport in rat adipose cells immediately after isolation, and then again after transfection (RSV-luc DNA, 0.2 µg/cuvette) and culture for 1 day (Fig. 4). Adding albumin to the incubation medium 2 hr after electroporation helped to preserve insulin responsiveness

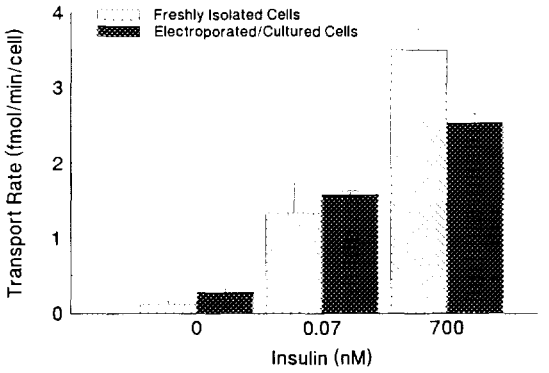

Figure 4. Insulin-stimulated 3-O-methylglucose transport in freshly isolated rat adipose cells vs. cells which were transfected with 0.2 μg RSV-luc DNA/cuvette and cultured for one day. The results shown are the means $\pm$ SEM of four experiments carried out using triplicate or quadruplicate samples.

with only a minimal decrease in transfection efficiency. In transfected, cultured cells, basal 3-O-methylglucose transport was approximately twice that of the freshly isolated cells. Maximal insulin stimulation of the transfected cells resulted in 3-O-methylglucose transport that was approximately 25% lower than the freshly isolated cells. Nevertheless, the transfected, cultured cells displayed a 10-fold response to maximal insulin stimulation (as compared with a 28-fold response in fresh cells). The luciferase activity measured in these transfected cells was comparable to that in our previous transfection experiments.

DISCUSSION

Electroporation is a common method for transfection of DNA into cultured cell lines (11). In freshly prepared rat adipose cells, electroporation has recently been used as a permeabilization method to examine the ability of small molecules such as H-7 and GTP-γS to alter cell function (12,13). These previous studies examined cell function within 30 min of electroporation. We have extended the use of electroporation in rat adipose cells to include transfection of DNA with high expression of a foreign protein and high insulin responsiveness after one day in primary culture.

During electroporation many variables can affect transfection efficiency and cell viability. For example, the voltage, capacitance, ionic strength and volume of the buffer, cuvette geometry, conduction characteristics and density of the cells, and the number of shocks are all critical variables (14). This is highlighted by the fact that we observed differences in transfection efficiency varying over three orders of magnitude with manipulation of only a few of these parameters. We observed an increase in luciferase activity which was related to the amount of DNA transfected and the number of shocks administered. This presumably relates

to the transfection efficiency. However, we also observed a negative correlation between cell survival (as assessed by insulin binding) and these factors. Thus, an optimal protocol needs to balance high transfection efficiency with minimal cell death and loss of function.

The luciferase reporter gene is well suited for assessing and optimizing transfection in adipose cells. The Promega assay, using a luciferyl-CoA substrate, has a linear range over 8 orders of magnitude and a light intensity which is stable for several minutes (15). This system has been estimated to be 100 times as sensitive as chloramphenicol acetyltransferase assays (16). It is possible to detect less than 10^{-20} moles under ideal conditions (15). Furthermore, since luciferase is an enzyme which is not found in normal adipose cells, the likelihood of false positive results is exceedingly low. The fact that we were unable to detect luciferase activity in cells transfected with pGEM7-luc (a promoterless vector), cells incubated in the presence of RSV-luc without electroporation, or cells which had been transfected with RSV-luc but only incubated for 3 hr also helps to rule out false positive results.

We used our transfection system to evaluate the ability of various promoters to function in adipose cells. The strongest promoter we tested contained the CMV promoter/enhancer with a generic intron in the 5' position (PCIS2-luc). Interestingly, the CMV promoter/enhancer alone (pcDNAI-luc) was unable to drive any detectable luciferase expression. The RSV long terminal repeat promoter (RSV-luc) and the SV40 early promoter with the HTLV-I long terminal repeat (SRα-luc) generated moderate luciferase activity. Similar to the CMV promoter, the SV40 early promoter alone (SV232A-luc) generated a barely detectable level of luciferase activity. Thus, even strong viral-based promoters such as CMV and SV40 seem to require additional sequences such as viral long terminal repeats or upstream introns in order to achieve high activity in adipose cells.

A major distinguishing feature of adipose cells is their ability to significantly increase glucose transport in response to insulin stimulation via translocation of GLUT4 from an intracellular pool to the cell surface. It has been reported that adipose cells in primary culture lose insulin responsiveness and GLUT4 expression after several days in culture (17,18). One important aspect of our method is that the transfected cells are capable of a ten-fold increase in glucose transport with maximal insulin stimulation at a time when the transfected DNA is being highly expressed (one day after electroporation). Thus, transfection of adipose cells may be useful for studying the signal transduction pathway involved in insulin-mediated glucose uptake.

Conclusions - We have developed a protocol for transfection of DNA into isolated rat adipose cells which results in high expression of the transfected DNA when driven by an appropriate promoter. This system is potentially useful for investigating adipose cell-specific promoters. In addition, since the transfected cells remain highly responsive to insulin, we

provide, for the first time, a useful method to apply transfection techniques to investigate the molecular mechanisms of insulin action in a *bona fide* insulin target cell in primary culture.

ACKNOWLEDGMENTS

We are grateful to Drs. Cornelia Gorman, Richard Roth, Charles Roberts, and Thierry Pineau for providing some of the DNA vectors used in this study. We thank Dr. Domenico Accili for critical reading of this manuscript.

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