Chronic Treatment with Insulin Selectively Down-regulates Cell-surface GLUT4 Glucose Transporters in 3T3-L1 Adipocytes*

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A new method for photoaffinity labeling of glucose transporters has been used to compare the effects of glucose-starvation, acute-insulin, and chronic-insulin treatments on the cell-surface glucose transporters in 3T3-L1 adipocytes. Starvation alone increased the cell-surface levels of GLUT1 and GLUT4 by ≈4- to 5-fold, respectively. As shown by Calender, D. M., Kitagawa, K., Tanner, L. T., Holman, G. D., and Lienhart, G. E. (1990) J. Biol. Chem. 265, 13800-13808) acute-insulin treatment increased cell-surface GLUT1 and GLUT4 by ≈5- and ≈15-fold respectively. In contrast to this, chronic-insulin treatment gave a further 3–4-fold increase in both cell-surface and total cellular GLUT1, but availability of GLUT4 at the cell-surface was down-regulated to half the level found in the acute treatment but with no change in the total cellular level. This effect occurred in starved and non-starved cells and suggests that starvation, acute-insulin, and chronic-insulin treatments regulate glucose transporter availability through independent mechanisms. The down-regulation of GLUT4 reached a maximally reduced cell-surface level in 6 h while the rise in GLUT1 reached a maximum after 24–48 h. The rise in GLUT1 appeared to compensate for the decline in cell-surface GLUT4 as glucose transport activity was further increased during the long-term treatment with insulin. The down-regulation of GLUT4 due to the chronic-insulin treatment is associated with a marked resistance of the cells to restimulate glucose transport and particularly to recruit further GLUT4 to the cell-surface following an additional insulin treatment. The defect appears to be in the signaling mechanism that is responsible for translocation.

Differentiated 3T3-L1 adipocytes are a good model system for investigating long-term regulatory effects on glucose transport. They respond to acute-insulin treatment with increases in glucose transport of 10-20-fold above basal levels (1, 2). It has recently been shown that differentiation of these cells from fibroblasts is associated with an increase in cellular mRNA and protein for the GLUT4 isoform (3, 4). Calderhead et al. (2) have shown that the cell-surface labeling of GLUT4 increases 15-fold above basal levels and that this increase is similar to, but slightly less than, the stimulation of glucose transport activity. The GLUT1 isoform is also abundant in differentiated 3T3-L1 cells (1, 2, 5), and this isoform increases 3-fold (1) to 5-fold (2) above basal levels in response to acute insulin. In contrast to this, chronic-insulin treatment has been shown (5, 6) to produce an 4-fold further increase in the mRNA and total protein for the GLUT1 isoform. However, no change was observed in the total cellular mRNA or protein for the GLUT4 isoform. The redistribution of these transporters to the cell-surface following the chronic treatment is examined here.

The relationship between glucose starvation and insulin treatments as modifiers of glucose transport has been examined in many cultured cells (6–11) including 3T3-L1 adipocytes (9–11) and in whole animal studies (12, 13). Starved rats show decreases in adipocyte GLUT4 which resemble those changes which occur in streptozotocin-treated diabetic rats. In both cases circulating insulin decreases and so separating the possible independent effects of insulin and starvation is difficult. An advantage of using cultured cells for studies on regulation of this type is that glucose-starvation and insulin treatment can be independently varied. A disadvantage of using cultured cells is that they generally contain high levels of the GLUT1 transporter which is only present at low levels in isolated adipose and muscle tissue. Thus, for the cultured cells a resolution of the separate changes occurring in GLUT1 and GLUT4 is required. It is particularly important to measure the cell-surface availability of these isoforms under the different regimes of starvation and acute or chronic-insulin treatment. We have therefore studied these changes here by cell-surface labeling the glucose transporters using the impermeant bis-mannose photolabel, 2-N-(1-azido-2, 2, 2-trifluoroethyl)benzyol-1,4-bis(d-mannos-4-xyloxy)-2-propylamine (ATB-BMPA) and then immunoprecipitating the labeled transporters with anti-GLUT1 or anti-GLUT4 antibodies (2, 14, 15).

We have also used this technique to examine changes in the cell-surface transporter distribution that are associated with the insulin resistance which has been shown to occur following a chronic-insulin treatment (16, 17).

EXPERIMENTAL PROCEDURES

Materials—ATB-2-(2-[3H]H)BMPA (specific activity ≈10 Ci/mmol) was prepared as described elsewhere (15). [1-2H]-Deoxy-d-glucose was from Amersham International. DMEM and RPMI were from Flow laboratories. Fetal bovine serum was from Gibco.

Cell Culture—3T3-L1 fibroblasts were cultured in DMEM and differentiated to adipocytes as described (1, 19, 20). Before use in 2-deoxy-d-glucose transport assays or in cell-surface labeling experiments the cells were subjected to a standard washing and refeeding procedure. Cells were first washed with phosphate-buffered saline (154 mM NaCl, 12.5 mM sodium phosphate, pH 7.4) and were then

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1The abbreviations used are: ATB-BMPA, 2-N-4-(1-azi-2, 2, 2-trifluoroethyl)benzyol-1,3-bis(d-mannos-4-xyloxy)-2-propylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MIES, 2-(N-morpholino)-ethanesulfonic acid; C6E6, nonaethyleneglycol dodecyl ether; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DME, Dulbecco’s modified Eagle’s medium.

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incubated for 2 h in serum-free medium containing 25 mM D-glucose. This was followed by three washes in Kreb’s-Ringer-HEPES buffer (KRH buffer, 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄, 10 mM HEPES, pH 7.4) and where appropriate with a subsequent treatment with 100 nM porcine monocomponent insulin for 30 min at 37 °C in 1 ml of KRH buffer (an acute-insulin treatment). In the case of the glucose-starvation treatment, fully differentiated cells were incubated in RPMI containing 25 mM D-xylene to replace glucose. In the case of the chronic-insulin treatment, fully differentiated cells were either incubated for 24 h in normal medium (DMEM with 25 mM D-glucose) or glucose-starvation medium (RPMI containing 25 mM D-xylene instead of D-glucose) with 500 nM porcine monocomponent insulin. In the cases where cells were glucose-starved and chronically insulin treated, the treatment was reversed using the standard washing and refedding procedure (above). For non-starved cells which were chronically insulin treated, the treatment was reversed by incubating cells for 1 h in Kreb’s-Ringer-MES buffer (of the same composition as KRH buffer) and where appropriate with MES instead of HEPES pH 6.0, and containing 25 mM D-glucose, followed by three further washes in this buffer in addition to the standard washing procedure (above).

2-Deoxy-D-Glucose Transport Assays—2-Deoxy-D-glucose uptake rates were determined as described (1, 19). The 3T3-L1 cell monolayers in 35-mm dishes were incubated with 50 μM 2-deoxy-[1-3H]D-glucose in 1 ml of KRH buffer at 37 °C for 5 min. Cells were then rapidly washed three times in KRH buffer at 0-4 °C and then the cell associated radioactivity was extracted and counted. The transport data were corrected for a background and for nonspecific uptake which was determined by incubation of cells in the presence of 30 μM cytochalasin B.

Insulin Binding—Tracer insulin binding was assayed as described by Van Putten et al. (18) with some modifications. Cells in 35-mm dishes were incubated in serum-free DMEM for 2 h, washed three times in KRH buffer, and incubated at 20 °C in 1 ml of KRH containing 70 fmol of mono-A14-125I-insulin, 2% serum albumin, bacitracin (10 mg/ml) and where appropriate with 5 nM insulin. The dishes were shaken (65 oscillations/min) for 3 h. After washing three times in KRH, 1 ml of ice-cold 10% trichloracetic acid was added and the insoluble protein precipitated and counted for radioactivity. Specific [125I]insulin binding was calculated as the difference between the 125I-insulin which was bound to the cells in the presence and absence of added unlabeled 5 nM insulin. The nonspecific binding was 25% of the total 125I-insulin binding. To determine insulin binding following chronic-insulin treatment, cells were subjected to the washing and refedding procedures described under “Cell Culture” above.

ATB-BMPA Photolabeling—Plates of differentiated cells (35 mm) were washed in KRH buffer and were then incubated in 200 μCi of ATB-[2H]BMPA for 2 min and irradiated for 1 min in a Rayonet RPR-100 photochemical reactor with RPR-300-nm lamps as described (2). To estimate relative changes in total cellular transporter, the differentiated cells were scraped from 35-mm dishes and homogenized in 500 μl of KRH buffer. The suspension was then mixed with 200 μCi of label in 55-mm dishes and irradiated as described for the cell-surface labeling. The irradiated cells or homogenates were then washed three times in KRH buffer and solubilized in 1 ml of detergent buffer containing 2% C₅₀E₅₀, 5 mM sodium phosphate, 5 mM EDTA, pH 7.2, with the protease inhibitors antipain, aprotinin, pepstatin, and leupeptin, each at 1 μg/ml. Following centrifugation at 20,000 × g for 20 min, the supernatants were subjected to sequential immunoprecipitation with 8 μl of protein A-sepharose coupled to either affinity purified anti-GLUT1 (20 μg) or anti-GLUT4 (10 μg) antibodies. These antibodies were raised against C-terminal peptides as described (14). After incubation for 1.5-2 h at 0-4 °C and washing of the immunoprecipitates three times with 0.2% C₅₀E₅₀ in detergent buffer, the labeled glucose transporters were released from the antibody complexes with SDS-urea electrophoresis sample buffer and subjected to electrophoresis on 10% acrylamide gels. The radioactivity on the gels was estimated by cutting and counting gel slices. The radioactivity in transporter peaks was corrected for a background which was based on the average radioactivity of the slices on either side of the peak (1).

RESULTS

Fig. 1 shows the acute response to insulin in non-starved 3T3-L1 cells. The ATB-BMPA was used to label intact cells, and the GLUT1 and GLUT4 immunoprecipitates were resolved on SDS-polyacrylamide gels. The acute (30 min) treatment with insulin increased the labeling of GLUT1 and GLUT4 in this experiment to ≈3- and ≈10-fold (≈3.5- and ≈12-fold from five experiments) of the level found with basal cells. Also shown in Fig. 1 is the effect of exposure of the cells to insulin for 24 h (a chronic treatment). This resulted in a further 4-fold increase in GLUT1 to give a total stimulation which was 13-fold above basal GLUT1 levels (≈14-fold from three experiments). In contrast to this, the cell-surface levels of GLUT4 were reduced to 53% of that found in the acute treatment (from three experiments). In addition we have confirmed (5) by measuring ATB-BMPA photolabeling of transporters in cell homogenates) that the total amount of GLUT1 rises 6-fold while the total amount of GLUT4 rises only slightly (by ≈1.4-fold) following the chronic-insulin treatment.

The time course for the up-regulation of GLUT1 and the down-regulation of cell-surface GLUT4 has been examined. Fig. 2 shows that a decrease in GLUT4 occurred after 4-6 h while GLUT1 steadily increased over 24 h and remained at this elevated level for a further 24 h. The stimulation of 2-
deoxy-D-glucose transport was increased by the chronic-insulin treatment, and Fig. 2 shows that this rise paralleled the increase in GLUT1.

Since long term regulation of glucose transport activity is known to be glucose sensitive (7-13) we also examined the acute and chronic responses to insulin in cells which were maintained in glucose-deprived media. This starvation regime resulted in a 3-fold rise in the basal level of 2-deoxy-D-glucose transport which as shown in Fig. 3 is associated with a ~4-fold increase in cell surface GLUT1 and a ~2-fold increase in cell-surface GLUT4 above basal levels obtained in non-starved cells from the same plating. As shown in this figure, the starvation regime reduces the fold change in insulin stimulation mainly by raising basal levels of cell-surface transporters. Chronic treatment of starved cells with insulin increased GLUT1 ~4-fold and decreases GLUT4 to 49% (from five experiments) of the levels found with the acute treatment. In both starved and non-starved cells, the ratio of GLUT1/GLUT4 changed from 1.1:1 in acute-insulin treatment to ~9:1 following chronic-insulin treatment.

In Fig. 4a and b, the 2-deoxy-D-glucose transport rates in non-starved and glucose-starved cells are compared with the cell-surface levels of photolabeled GLUT1 and GLUT4. The results presented in this figure are the mean and S.E. from three to seven independent experiments. In non-starved cells acute-insulin treatment increased the 2-deoxy-D-glucose transport by ~14-fold compared with basal levels. Chronic-insulin treatment of both non-starved and glucose-starved cells resulted in only an additional 40% increase in glucose transport activity above that found following the acute treatment. The very large 4-fold increase in GLUT1 compensates for a ~50% down-regulation of cell-surface GLUT4.

We next examined whether the down-regulation of the GLUT4 glucose transporter in both starved and non-starved cells is associated with any lesion in the cell's ability to respond to a subsequent acute-insulin treatment (Figs. 4 and 5). In Fig. 4, a and b, the 2-deoxy-D-glucose transport rates are compared with the labeling of both GLUT1 and GLUT4. In all cases the second challenge with insulin resulted in blunted responses. Fig. 5 shows that the effect of starvation alone is readily reversed by refeeding with 25 mM D-glucose. The starved and refed cells showed normal rises in cell-surface GLUT1 (panel a) and GLUT4 (panel c) in response to acute-insulin treatment. However, starved cells which had been

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**Fig. 2.** Time course for the changes in cell-surface transporters during chronic-insulin treatment. Differentiated cells in 35-mm dishes were treated with 500 nM insulin for 30 min to 48 h and then glucose transporters were cell-surface labeled with 200 μCi of ATB-[2-3H]BMPA and were immunoprecipitated with GLUT1 (O) or GLUT4 (●) anti-C-terminal-peptide antibodies as described under "Experimental Procedures." The labeling of each transporter was estimated from SDS-PAGE gel slices and the results are expressed as a percentage of the acute (30 min) treatment with insulin. Changes in cell-surface transporters are compared with 2-deoxy-D-glucose transport rates obtained from the same cell plating (●).

**Fig. 3.** The effect of acute- and chronic-insulin treatments on cell-surface-labeled glucose transporters in glucose-starved 3T3-L1 adipocytes. Differentiated cells in 35-mm dishes were glucose starved for 24 h in RPMI medium containing 25 mM D-xylose to replace glucose. The glucose transporters were cell-surface labeled with 200 μCi of [2-3H]BMPA and were immunoprecipitated with GLUT1 or GLUT4 anti-C-terminal-peptide antibodies as described under "Experimental Procedures." The SDS-PAGE gel profile for basal (O), acute-insulin (●) treatment (100 nM for 30 min), and chronic-insulin (▼) treatment (500 nM for 24 h) are shown.
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Fig. 4. The effect of chronic-insulin treatment of 3T3-L1 adipocytes on the ability to respond to a subsequent acute-insulin stimulation: a comparison of 2-deoxy-D-glucose transport activity and cell-surface-labeled transporters. In a, fully differentiated non-starved cells in 35-mm dishes which were B (basal), IA (acutely insulin treated), and IC (chronically insulin treated) were subjected to 2-deoxy-D-glucose transport assays or cell-surface labeling of GLUT1 and GLUT4 transporters as described under "Experimental Procedures." Some of the chronically insulin-treated cells were washed to remove insulin and incubated in medium containing 25 mM D-glucose as described under "Experimental Procedures" (ICR or chronic-insulin reversed cells) and were then tested for response to a further acute-insulin treatment (ICA). In b, fully differentiated glucose-starved cells in 35-mm dishes were treated in the same way as described in a and were then subjected to analysis of 2-deoxy-D-glucose transport and levels of cell-surface GLUT1 and GLUT4 as described under "Experimental Procedures." The results show the mean and S.E. estimated from three to seven separate experiments.

treated chronically with insulin and then washed and refed in an insulin-free buffer showed very small increases in these cell-surface transporters in response to further insulin treatment (panel b and d). The transport stimulations in response to the second insulin treatment were also found to be reduced below those found in a normal acute insulin response (Fig. 4b), but the increases in transport appeared to be greater than could be accounted for by any increase in cell-surface transporters. Thus, these cells have developed striking insulin resistance mainly in the ability to recruit additional transporters to the cell-surface. In addition, it is clear from this experiment that starvation alone is not responsible for this resistance. We have confirmed (17) that insulin binding is unchanged following chronic-insulin treatment; the tracer insulin binding was 88% (for starved cells) and 88% (for cells which had been subjected to the starvation and chronic insulin and washing regime) of that of normal cells.

In non-starved chronically insulin-treated cells, the transport stimulation due to chronic insulin is more persistent and is more difficult to reverse. The resistance to further insulin stimulation thus has to be measured against an already high transport rate. The persistence of the elevated transport activity appears to be due to a failure to reverse the high cell-surface level of GLUT1 transporters. As is the case in starved cells, the non-starved cells show a larger proportionate increase in 2-deoxy-D-glucose transport activity in response to additional insulin than can be accounted for by any additional increase in cell-surface transporters. As in non-starved cells, the main source of the resistance appears to be in a failure to recruit additional transporters to the plasma membrane.

DISCUSSION

The bis-mannose-photolabeling technique has been shown to provide a means of studying changes in cell-surface levels of glucose transporters without the need to subcellular fractionate cells (2, 14). The acute effect of insulin on cell-surface labeled transporters has been studied in 3T3-L1 cells (2) and in rat adipocytes (14). In both systems insulin stimulates the
chloride in the presence of 2-D-glucose under "Experimental Procedures." Following cell-surface labeling with 200 μCi of ATB-[3-H]BMPA, the GLUT1 isoform (panel a) and the GLUT4 isoform (panel c) were immunoprecipitated and subjected to electrophoresis. Starved cells which were chronically treated with insulin and then washed and refed with glucose as in a and were then incubated with (●) or without (○) acute insulin. Following the cell-surface labeling with ATB-BMPA, the GLUT1 isoform (panel b) and the GLUT4 isoform (panel d) were immunoprecipitated and subjected to electrophoresis.

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Fig. 5. A comparison of the cell-surface labeling of glucose transporters in starved 3T3-L1 adipocytes before and after chronic-insulin treatment. Glucose-starved cells without chronic-insulin treatment were re-fed with 25 mM d-glucose for 2 h at 37°C and then incubated with (●) or without (○) acute insulin as described under "Experimental Procedures." Following cell-surface labeling with 200 μCi of ATB-[3-H]BMPA, the GLUT1 isoform (panel a) and the GLUT4 isoform (panel c) were immunoprecipitated and subjected to electrophoresis. Starved cells which were chronically treated with insulin and then washed and refed with glucose as in a and were then incubated with (●) or without (○) acute insulin. Following the cell-surface labeling with ATB-BMPA, the GLUT1 isoform (panel b) and the GLUT4 isoform (panel d) were immunoprecipitated and subjected to electrophoresis.

cell-surface labeling of GLUT4 by 15-fold while GLUT1 is increased by only 3-fold. Translocation of GLUT4 to the cell-surface can therefore account for a large proportion of the stimulatory effect of insulin on glucose transport activity in these cells. However, the stimulation of transport activity is generally slightly greater (2-fold) than the translocation of either transporter so that intrinsic activation of these transporters may also occur as suggested (21-24).

As well as providing a means of measuring changes in each transporter, the technique can also give an estimate of the relative abundance of the two transporter isoforms. These latter comparisons are of course only valid if the efficiencies of labeling and immunoprecipitation of the two transporters are equal. Western blotting has shown that erythrocytes contain only GLUT1 (25) and that in rat adipocytes over 90% of the total transporter is the GLUT4 isoform (2, 26-28). The affinities of GLUT1 (in erythrocytes) and GLUT4 (the predominant isoform in rat adipocytes) for ATB-BMPA have been shown, from transport inhibition experiments, to be 250-300 μM (14, 15). We have measured the K_i for ATB-BMPA inhibition of 3-O-methyl-D-glucose transport in 3T3-L1 cells and find that this is 250 μM following both acute and chronic-insulin treatments. In addition, we have carried out binding experiments at a range of ATB-BMPA concentrations and find that the K_i of GLUT1 and GLUT4 are equal and are 200 μM following both acute- and chronic-insulin treatments. The efficiencies of labeling of these glucose transporters in erythrocytes and adipocytes has been estimated to be 2500 dpm/mmol transporter/mCi ml^-1 of ATB-BMPA (14). These comparisons suggest that the labeling efficiencies for GLUT1 and GLUT4 are roughly equal. In addition, we have shown that the immunoprecipitations of erythrocyte GLUT1 and adipocyte GLUT4 are about 70-80% efficient (14, 15). Thus, the proportion of label recovered in the GLUT1 and GLUT4 immunoprecipitates is likely to be a good approximation of the relative abundance of the two isoforms. In insulin-stimulated rat adipocytes, our estimate (14) of the ratio of GLUT1/GLUT4 is 1:10 which agrees well with the Western blotting data of total cell membranes (2). However, in insulin-stimulated 3T3-L1 cells the relative abundance of the GLUT1/GLUT4 as estimated from cell-surface labeling with ATB-BMPA is 1.6:1 (2) while Western blotting of total cell membranes has shown a ratio of 3:1 (2). However, Western blotting data is also subject to quantitative uncertainties such as the reliability of the protein standards. The transporter contents of these protein standards have to be equated with the number of cytochalasin B-binding sites which may also be uncertain since the affinity of GLUT4 for cytochalasin B has not been directly measured. Thus, both techniques may give only an approximation of the relative transporter abundance but can give a reliable estimate of changes in the levels of each transporter.

Tordjman et al. (5, 11) have examined the long term effects of starvation and of chronic-insulin treatment on glucose transport regulation in 3T3-L1 cells by Western blotting of the GLUT1 and GLUT4 isoforms. The increases in ATB-BMPA-labeled cell-surface GLUT1 (by 4-fold) and GLUT4 (by 2-fold) following starvation that we have observed here agree with their results (11). Tordjman et al. (5) and Hanique et al. (6) showed that chronic-insulin treatment increased total cellular GLUT1 by 4-fold but did not change total cellular GLUT4. We have confirmed this in the cells used in our study by photolabeling the total cell homogenates with ATB-BMPA (see "Results") and by photolabeling transporters in digitonin-permeabilized cells with ATB-BMPA. However, Clancy and Czech (24) have recently reported a reduction in total cellular GLUT4 following chronic-insulin treatment that we have excluded in these studies on the total cell homogenates by showing that GLUT4 at the cell-surface is down-regulated following the chronic-insulin treatment while levels of GLUT1 at the surface are markedly increased. The down-regulation occurs before the increase in GLUT1 at the surface reaches a maximum. Over this time course glucose transport activity is also increased. This suggests that the increase in GLUT1 compensates for the decrease in GLUT4. The effect occurs in non-starved and glucose-starved cells and suggests that the two transporters are independently regulated by glucose and insulin.

The chronic-insulin treatment has been shown here and by Rosen et al. (17) to cause a persistent lesion in the cells ability to increase glucose transport activity in response to further acute-insulin treatment. We show here that the treatment is particularly associated with a resistance of the cells to recruit further GLUT4 to the cell-surface following the additional insulin treatment. 2-Deoxy-D-glucose transport activity has been shown to be less resistant than transporter recruitment to the second insulin challenge. This suggests that, in addition to the well established role of transporter translocation in activation of transport (29, 30), intrinsic activation of any transporters already present in the plasma membrane may
also play an important role in regulation of the glucose transport rate (21–24). Thus, it is possible that when insulin is removed there is some intrinsic deactivation of GLUT1 transporters that are not removed from the surface. These cell-surface transporters may be reactivated in the subsequent insulin-resistant state. However, the most serious lesion that occurs following chronic-insulin treatment of starved and non-starved cells appears to be a failure to increase GLUT4 to normal cell-surface levels in a subsequent acute-insulin challenge.

Three types of insulin resistance in glucose transport have been recognized (31–38). These are resistance at the insulin receptor level (31–33), resistance in the signaling pathway (34, 35), and resistance due to a depleted pool of glucose reserves of transporter. Instead, chronic-insulin treatment results in a failure to increase GLUT4 and the subsequent resistance in the signaling pathway between the occupied insulin receptor and the glucose transporter. We also thank Professor R. M. Denton and Dr. G. W. Gould for 3T3-L1 cells and for their advice particularly concerning the culture of this cell line.

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