Insulin resistance is a common clinical feature of obesity and non-insulin-dependent diabetes mellitus, and is characterized by elevated serum levels of glucose, insulin, and lipids. The mechanism by which insulin resistance is acquired is unknown. We have previously demonstrated that upon chronic treatment of fibroblasts with insulin, conditions that mimic the hyperinsulinemia associated with insulin resistance, the membrane-associated insulin receptor β subunit is proteolytically cleaved, resulting in the generation of a cytosolic fragment of the β subunit, β', and that the generation of β' is inhibited by the thiol protease inhibitor E64 (Knutson, V. P. (1991) J. Biol. Chem. 266, 15656–15662). In this report, we demonstrate that in 3T3-L1 adipocytes: 1) cytosolic β' is generated by chronic insulin administration to the cells, and that E64 inhibits the production of β'; 2) chronic administration of insulin to the adipocytes leads to an insulin-resistant state, as measured by lipogenesis and glycolysis; and E64 totally prevents the generation of this insulin-induced cellular insulin resistance; 3) E64 has no effect on the insulin-induced down-regulation of insulin receptor substrate-1, and therefore insulin resistance is not mediated by the down-regulation of insulin receptor substrate-1; 4) under in vitro conditions, partially purified β' stoichiometrically inhibits the insulin-induced autophosphorylation of the receptor β subunit; and 5) administration of E64 to obese Zucker fatty rats improves the insulin resistance of the rats compared to saline-treated animals. These data indicate that β' is a mediator of insulin resistance, and the mechanism of action of β' is the inhibition of the insulin-induced autophosphorylation of the β subunit of the insulin receptor.

Insulin resistance is a characteristic clinical feature of a number of disease states, chief among them diabetes mellitus, and is associated with hyperglycemia, hyperinsulinemia, hyperlipidemia, and hypertension (reviewed in Refs. 1 and 2). The potential mechanisms by which cellular insulin resistance is generated are many: a mutation in the gene coding for the insulin receptor protein, resulting in a decreased expression of the protein; a decrease in the binding of insulin; a decrease in the number of insulin receptor molecules expressed on the plasma membrane of the target cell; or a so-called “post-receptor defect” in which there is a decreased interaction between the insulin receptor and downstream effector molecules.

Natural mutations in the primary sequence of the insulin receptor have been identified in patients with extreme forms of insulin resistance (3, 4). Mutations in the extracellular ligand binding domain of the receptor have been shown to result in a decreased affinity of insulin for the receptor (5–9). Mutations have also been documented in the intracellular β subunit of the receptor, especially in the ATP binding domain and the autophosphorylation domain of the receptor protein, resulting in a decreased tyrosine kinase activity of the receptor (10–12). However, of the many individuals who demonstrate insulin resistance, only a small number of them have been shown to have mutations in the primary sequence of the insulin receptor protein (13–17). Therefore, sequence abnormalities in the insulin receptor protein cannot alone account for insulin resistance, and is not, therefore, a frequent mechanism leading to the insulin-resistant state (2).

Insulin resistance induced by a decrease in receptor number could occur as a result of changes in the mRNA levels for the protein, a decreased efficiency of post-translational processing of the newly synthesized receptor protein, or a down-regulation of the level of the receptor in the cell. A decrease in the cellular levels of insulin receptor mRNA has been documented in a number of cases (18–22), with concomitant decreases in the level of insulin receptor protein. A decreased rate of insertion of the insulin receptor into the plasma membrane has also been demonstrated (10–12), primarily as a result of mutations in the primary sequence of the insulin binding a subunit of the receptor. An elevated rate of degradation of the insulin receptor protein, resulting in a decreased steady-state level of cellular receptor, has also been found to be due to specific modifications in the primary sequence of the receptor (5, 6). However, an extremely consistent finding, both in vivo, in hyperinsulinemic animals models and humans, and in cultured cells, is that the steady-state level of the insulin receptor is decreased by chronic exposure of the cells and tissues to insulin (23–26). In cultured cells, this down-regulation of the insulin receptor has been demonstrated to be due to an accelerated rate of receptor inactivation and degradation (27, 28). Therefore, a significant degree of insulin resistance could be explained by the insulin-induced down-regulation of the insulin receptor.

Modifications in the insulin signal transduction pathway distal to the actual binding of insulin to the receptor could also account for the insulin-resistant state. These processes could be brought about by a change in the insulin-induced autophosphorylation of the insulin receptor β subunit, a decrease in the tyrosine kinase activity of the receptor, or a decrease in the cellular level of the substrates of the insulin receptor tyrosine kinase. A significant body of work has documented that com-
pared to normal patients, in insulin-resistant patients with non-insulin-dependent diabetes mellitus, insulin binding to the receptor rapidly (14). The reduced level of tyrosine phosphorylation in the major catalytic domain of the receptor. This decreased autophosphorylation subsequently leads to the decreased activation of the receptor tyrosine kinase activity (29–32). Decreased levels of the coupling molecules or effector molecules in the insulin signal transduction pathway could also account for insulin resistance. Multiple substrates of the insulin receptor tyrosine kinase have been identified (33). One of them is a non-insulin-dependent diabetes mellitus, insulin binding to the receptor regulates the cellular levels of IRS-1 and IRS-2, which mediate in the insulin signal transduction pathway. IRS-1 levels were maintained in down-regulated state, although IRS-1 levels are maintained in a downregulated state. Furthermore, administration of IRS-1 to Zucker fatty rats results in a decrease of plasma triglyceride levels compared to control animals, and a decrease in the expression of IRS-1 in the tissues of the obese treated rats.

In vitro experiments with cultured fibroblasts with insulin, the cellular level of the intact insulin receptor protein decreases, and a fragment of the insulin receptor β subunit is produced and released from the cellular membranes into the cytosol of the cell (38). In this report, we demonstrate that this fragment, β', is also produced in cultured adipocytes, and that inhibition of the generation of β' with the thiol protease inhibitor E64 results in the total abrogation of insulin-induced insulin resistance in cultured adipocytes, even though IRS-1 levels are maintained in a downregulated state. Furthermore, administration of E64 to Zucker fatty rats results in a decrease in plasma triglyceride levels compared to control animals, and a decrease in the expression of β' in the tissues of the obese treated rats. In vitro, partially purified β' inhibits the insulin-induced autophosphorylation of the insulin receptor. These data indicate that β' mediates insulin resistance by inhibiting the autophosphorylation of the insulin receptor.

**EXPERIMENTAL PROCEDURES**

Materials—Cell culture media and sera were purchased from J RH (Kansas City, MO). Tissue culture plastic was purchased from Falcon (Becton Dickinson (Lincoln Park, NJ)). Purified insulin was obtained from Eli Lilly (Indianapolis, IN). Carrier-free Na32P3O10 (3000 Ci/mmol), Hypervilix x-ray film, and the enhanced chemiluminescence detection reagents (ECL) for immunoblot analysis were purchased from Amersham Corp. c-13C(luciferin)32P(300 Ci/mmol) was obtained from DuPont NEN. Antibodies against IRS-1 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Magnetized beads for conjugation to antibodies (Dynabeads M-450) were obtained from Dynal Inc. (Great Neck, NY). Female obese Zucker fatty rats (fa/fa), or age-matched lean Zucker rats (fa/+/) were obtained from Charles River Laboratories (Boston, MA). Serum glucose levels were quantitated by the glucose oxidase method with a kit purchased from Sigma Diagnostics. Blood triglyceride levels were quantitated spectrophotometrically with reagents obtained from Boehringer Mannheim Diagnostics. Insulin levels were quantitated by radioimmunoassay with a kit obtained from Bio-Rad. Biochemicals were obtained from Sigma or Calbiochem unless otherwise noted. All other reagents were of analytical grade or better and were obtained from common supply houses.

Cell Culture—Embryonic mouse 3T3-L1 pre-adipocytes were cultured in Dulbecco’s modified Eagle’s media containing 10% charcoal-filtered calf serum, in a humidified atmosphere of 10% CO2, as described previously (39). The cells were induced to differentiate into adipocytes and were subsequently maintained in the differentiated phenotype as described previously (28). All experiments were performed with cell monolayers whose cell culture media had been changed 18–24 h before the beginning of the experiment.

Preparation of Intact Insulin Receptor and Insulin Receptor Fragment—Cellular membranes containing the intact insulin receptor and cellular cytosol containing the fragment of the insulin receptor β subunit, β', were prepared from 10-cm dishes of fully differentiated 3T3-L1 adipocytes, as we have previously described for 3T3-C3 fibroblasts (38). Briefly, monolayers were preincubated with 1.7 μM insulin in complete cell culture media for the indicated periods of time; monolayers were then removed from the CO2 incubator, washed with phosphate-buffered saline at 4°C, and scraped into cell lysis buffer (50 mM Tris, pH 7.4, at 4°C, containing the following protease inhibitors: leupeptin, 1 μg/ml; pepstatin A, 1 μg/ml; chymostatin, 1 μg/ml; benzamidine, 10 μg/ml; phenylmethylsulfonyl fluoride, 0.2 mM; E64, 5 μg/ml; EDTA, 10 mM). Insulin was immunoprecipitated in a glass homogenizer with a tight-fitting, magnet-driven Teflon pestle, and the homogenate was subjected to centrifugation at 200,000 × g for 45 min. The fat cake was discarded, the infranatant was utilized as the cytosolic fraction, and the pellet contained the cellular membranes.

The pellet was extracted in cell lysis buffer containing 4% Triton X-100. Following homogenization of the pellet, the sample was incubated on ice for 1 h to optimize extraction of the membrane proteins, and then subjected to centrifugation at 200,000 × g for 45 min. Following removal of any residual fat cake, this supernatant contained the extracted membrane proteins, including the intact insulin receptor.

The cytosolic fraction contained β'. This crude cytosolic fraction was either utilized directly for immunoblot analysis of β' or IRS-1 (see below) or was further processed in an isoelectric focusing. Preparative isoelectric focusing was performed in the Rotofor apparatus (Bio-Rad), over a pH range of 2–12. The crude cytosol was added to 2 ml of 40% ampholytes, and the mixture was subjected to focusing in the cooled chamber, as described by the manufacturer. During the isoelectric focusing, the temperature was maintained at approximately 5°C. Following focusing, the gradient was fractionated into 20 fractions. The pH of the fractions was determined, and an aliquot of each fraction was subjected to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis to localize β' (see below). Fractions containing β' were pooled and utilized in the insulin receptor autophosphorylation assay (see below).

Immunoblot Analysis—Samples were subjected to SDS-PAGE on discontinuous 7.5% gels, transferred to nitrocellulose, and probed with the antibodies, as described previously (40). The antibody used to detect the β subunit of the insulin receptor and β' is denoted anti-P5 antibody, and was utilized at a concentration of 5 μg/ml. The antibody against IRS-1 was utilized at a concentration of 4 μg/ml. Enhanced chemiluminescence was used to detect the immunoreactive bands, as described by the manufacturer. In order to reprobe a membrane with a second primary antibody, the nitrocellulose membranes were stripped by incubation with 75 mM dithiothreitol in 50 mM Tris, 200 mM NaCl, pH 8, with agitation. After 1 h, this buffer was removed from the membrane, NaSCN was added to the solution to a concentration of 3 M, and incubation was continued for an additional 18 h. Subsequent detection of antibody bound to the membrane by enhanced chemiluminescence demonstrated no residual signal, and no apparent loss of immobilized proteins by the stripping process for a total of three rounds of stripping and reprobing (data not shown). Quantitation of the immunoblots was performed by densitometry with a Biomage 605 (Milligen/Bioresearch, Division of Millipore).

Insulin-induced Lipogenesis, Glycogen Synthesis, and Glucose Uptake—Lipogenesis and glycogen synthesis, utilizing [14C]glucose as a substrate, and glucose uptake, monitoring the rates of uptake of [3H]deoxyglucose, were performed as described previously. Briefly, fully differentiated 3T3-L1 adipocytes in 6-cm culture dishes were preincubated with 1.7 μM insulin in the presence or absence of E64 for 0–18 h, as indicated in the legends to the figures. Following the preincubation, the monolayers were exhaustively washed with Kreb’s-Ringer-phosphate buffer containing 25 mM glucose and 2% bovine serum albumin (radioimmunoassay grade) over a 1-h period. We previously demonstrated that this wash procedure removes all insulin and insulin degradation products from the cell monolayers (27, 28). Following this wash procedure, the cell monolayers were stimulated with 0–25 mM insulin in 3 ml of Kreb’s-Ringer-phosphate buffer for 20 min at 37°C. To each dish was then added the radiolabeled substrate, and the incubation was allowed to proceed for an additional 20 min. Incorporation of radiolabel into cellular lipids and cellular glycogen was a linear function of time from 0 to at least 30 min (data not shown). Uptake of d}

1 The abbreviations used are: IRS-1, insulin receptor substrate-1; PAGE, polyacrylamide gel electrophoresis.

2 V. P. Knutson, Y. Balba, and T. Elliott, submitted for publication.
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A constant amount of insulin receptor was incubated with graded concentrations of β' for 10 min before the addition of 100 μM insulin or buffer alone. After incubation for 20 min at room temperature, \( [\gamma-^{32}P]ATP \) was added to the assay at a final concentration of 15 μM, 20 μCi/assay (170 μl total volume). The final assay contained 50 mM Tris, pH 7.4, 5 mM MnCl\(_2\), 5 mM MgCl\(_2\), 2 mM molybdate, 0.1% ovalbumin, and 0.1% Triton X-100. The assay was terminated after 1 h by the addition of SDS-PAGE sample buffer and boiling for 5 min. The samples were subjected to SDS-PAGE as described above, on two replicate gels. One gel was stained, destained, and dried. Radioactivity associated with the insulin receptor β subunit (92 kDa) and β' (61 kDa) was quantitated with a Betagen Betascope 603 blot analyzer. The proteins in the second gel were transferred to nitrocellulose and subjected to immunoblot analysis with the antibody against the insulin receptor β subunit. The relative proportions of β subunit and β' were calculated from the immunoblot by densitometry.

Zucker Fatty Rat Protocol—Six female obese (fa/fa) and six female lean (Fa?) rats were obtained from the breeder at the age of 12 weeks. The animals were fed ad libitum and maintained on 12-h light/dark cycles. Over the following 2-week period, blood was drawn from the tail artery of the animals under mild ether anesthesia, and the levels of insulin, glucose, and triglycerides were quantitated. At the age of 14 weeks, when the insulin resistance of the animals was fully manifest, the E64 protocol was initiated. E64 was dissolved in saturated sodium bicarbonate and diluted with normal saline. Three fatty rats and three lean rats were injected intraperitoneally with E64 (2.5 mg/kg) in a total volume of 0.3 ml, every 24 ± 2 h. Three fatty rats and three lean rats received vehicle alone. Before collecting blood samples, the rats were fasted for approximately 4 h. Following collection from the tail artery, the blood was allowed to clot, and serum was collected. Aliquots for triglyceride analysis were collected and stored at −20 °C. The remaining serum was subjected to centrifugation at 20,000 × g for 40 min at 4 °C. This procedure resulted in the formation of a fat cake at the top of the sample. The infranatant was collected and stored at −20 °C for insulin and glucose determinations. Blood was collected on the following days, where day 1 was the first injection day: −15, −9, −4, 2, 5, 10, 14, and at 7-day intervals thereafter, up to and including day 89.

Statistical significance was determined by Student’s t test; where appropriate, p values are indicated in the legends to the figures.

RESULTS

To verify in 3T3-L1 adipocytes the precursor-product relationship between the insulin receptor β subunit and the cytosolic fragment β', and to verify that E64, the irreversible thiol proteinase inhibitor, inhibits the proteolysis of intact β subunit, 3T3-L1 adipocytes were treated with 1.7 μM insulin for 0, 5, or 18 h in the absence or presence of E64. Immunoblot analysis of the membrane fraction of 3T3-L1 adipocytes with the anti-P5 antibody (specific for the carboxyl terminus of the β subunit of the insulin receptor) is shown in Fig. 1A, and densitometric analysis of the blot is shown in Fig. 1B. After 5 h of insulin incubation (−E64, lanes 3 and 4), the intensity of the intact, 92-kDa β subunit of the insulin receptor is decreased by approximately 35% compared to 0 h of insulin treatment (lanes 1 and 2). By 18 h of insulin treatment, intact β subunit is even further decreased by approximately 67% (+E64, lanes 7 and 8). This insulin-induced loss of immunodetectable β subunit is consistent with the insulin-induced loss of insulin binding activity we have previously reported in 3T3-L1 adipocytes (28). When 100 μM E64 was added simultaneously with the insulin, the E64 inhibited the insulin-induced loss of membrane-associated intact β subunit at 5 h (+E64, lanes 5 and 6; compare with control lanes 1 and 2). After coincubation of both insulin and E64 for 18 h (+E64, lanes 9 and 10), E64 was only partially effective in inhibiting the insulin-induced down-regulation of the β subunit. Therefore, E64 inhibited the insulin-induced loss of β subunit from the cellular membranes, but the effectiveness of E64 was lost with the prolonged, 18-h incubation.

Fig. 1C demonstrates a representative immunoblot of the effect of insulin and E64 on the generation of the cytosolic fragment of the insulin receptor β subunit, β', with densitometric analysis of data from two separate experiments in Fig. 1D. As demonstrated in lanes 1 and 2, prior to treatment of the cells with insulin, the level of the 61-kDa β' in the cytosol of the cells is low. However, after 5 h of insulin treatment (−E64, lanes 3 and 4), the level of β' increased 2-fold above basal, and after 18 h of insulin treatment of the cells (−E64, lanes 7 and 8), the level of β' was increased approximately 3.5-fold above basal levels. Simultaneous treatment of the 3T3-L1 adipocytes with both insulin and E64 resulted in the inhibition of the production of β' in the cytosol after 5 h of treatment (+E64, lanes 5 and 6). In fact, in some experiments, the level of β' detected upon coincubation with both insulin and E64 was less than the level of β' found in the control cells. After 18 h of coincubation with both E64 and insulin (+E64, lanes 9 and 10), elevated levels of β' were demonstrated, again indicating that the E64 was losing its potency in inhibiting the insulin-induced production of the fragment. Therefore, E64 prevented the proteolysis of the insulin receptor β subunit induced by insulin, and this effect was readily demonstrated after 5 h of incubation with both insulin and E64. However, the effect of E64 in 3T3-L1 adipocytes was transient, and the cells recovered from the inhibition by E64 by 18 h.

The data described above indicate that E64 inhibited the insulin-induced loss of the insulin receptor β subunit from the cellular membranes. Since down-regulation of the insulin receptor is associated with insulin resistance, it was of interest to assess the effect of E64 on insulin-induced insulin resistance in 3T3-L1 adipocytes. Three indices of insulin sensitivity and insulin resistance were assessed: lipogenesis, glycogen synthesis, and glucose uptake.

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Fig. 1. The effect of E64 on the insulin-induced loss of intact insulin receptor β subunit from membranes of 3T3-L1 adipocytes, and the insulin-induced production of β' in the cellular cytosol. 3T3-L1 adipocytes were treated with 1.7 μM insulin for 0, 5, or 18 h in the presence or absence of 100 μM E64. Intact insulin receptor was extracted from the total membrane pellet of the cells, and β' was obtained from the cellular cytosol, as described under “Experimental Procedures.” Panel A, immunoblot analysis of intact β subunit of the insulin receptor. Membrane extracts were from cells treated with insulin for 0, 5, or 18 h (time of incubation) in the absence (−E64) or presence (+E64) of the thiol protease inhibitor. Samples from each experimental condition were subjected to SDS-PAGE in duplicate. Following SDS-PAGE and transfer to nitrocellulose, the transfer was incubated with the anti-insulin receptor antibody, as described under “Experimental Procedures.” Panel B, densitometric analysis of the immunoblot in panel A. Solid gray bars, insulin treatment alone for the indicated incubation time. Cross-hatched bars, insulin plus E64 treatment for the indicated incubation time. Panel C, immunoblot analysis of β' from the cytosol of treated cells. Cytosol from cells treated with insulin for 0, 5, or 18 h (incubation time) in the absence (−E64) or presence (+E64) of E64. Samples from each experimental condition were subjected to SDS-PAGE in duplicate. Following SDS-PAGE and transfer to nitrocellulose, the transfer was incubated with the anti-insulin receptor antibody, as described under “Experimental Procedures.” Panel D, densitometric analysis of the immunoblot in panel C. Solid gray bars, insulin treatment alone for the indicated incubation time. Cross-hatched bars, insulin plus E64 treatment for the indicated incubation time. Each sample subjected to SDS-PAGE, either membrane extract or cytosol, was obtained from approximately 1 × 10^6 cells.

The effect of E64 on glycogen synthesis was also explored. We had previously demonstrated that insulin stimulation of 3T3-L1 adipocytes induces a robust 14-fold induction of glycogen synthesis, with half-maximal induction occurring at approximately 5 nM insulin. As with lipogenesis, chronic insulin pretreatment of the cells resulted in an increase in basal glycogen synthesis, but a total loss of insulin-inducible glycogen synthesis. Chronic insulin pretreatment induces insulin resistance of glycogen synthesis. These results are recapitulated in Fig. 2 (panel B), demonstrating a 14-fold induction in control (C) cells and insulin resistance in insulin-pretreated cells (9E and 18E). The effect of E64 on glycogen synthesis was then determined, and data on the coinoculation of the cells with both insulin and E64 for 9 or 18 h are also shown in Fig. 2B. The induction of 100 mM E64 in the 9- and 18-h insulin incubation (9E and 18E) resulted in an improvement of insulin sensitivity of the cells, compared to cells incubated with insulin alone. After both 9 and 18 h of preincubation with both insulin and E64, acute insulin stimulation could induce a 2-fold increase in glycogen synthesis. Unlike the results with lipogenesis, E64 treatment did not decrease the basal rates of glycogen synthesis to control cell levels (compare C (0 nM insulin) with 9E (0 nM insulin) and 18E (0 nM insulin)), and the maximal rates of glycogen synthesis in the E64-treated cells were not as high as
the rates demonstrated in the control cells (compare C (25 nm insulin) with 9EI (25 nm insulin) and 18EI (25 nm insulin)). However, insulin responsiveness was clearly apparent in the E64-treated cells but absent in the cells not treated with E64 (compare 9I (25 nm insulin) with 9EI (25 nm insulin) or 18I (25 nm insulin) with 18EI (25 nm insulin)). Therefore, incubation of the 3T3-L1 adipocytes with both insulin and E64 preserved the insulin responsiveness of the cells. The effect of E64 on glucose uptake was also pursued. We had previously demonstrated that insulin stimulates glucose uptake into 3T3-L1 adipocytes 8-fold, and that chronic insulin treatment of the cells induces an insulin-resistant state by elevating basal glucose uptake with a concomitant reduction in insulin stimulated uptake to approximately 2-fold. These data are recapitulated in Fig. 2C, where control cells (C) demonstrate an 8-fold induction of glucose transport, with half-maximal uptake demonstrated at 2.5 nm insulin, and cells treated with insulin for 9 (9I) or 18 (18I) h demonstrate elevated basal rates of uptake and very little additional insulin-induced uptake. The effects of E64 on glucose uptake were then determined, and these data are also presented in Fig. 2C. In marked contrast to the data presented in panels A and B, incubation of the adipocytes with E64 did not preserve the insulin-responsiveness of the glucose transport process in these cells. After 9 h of insulin pretreatment, basal transport was elevated approximately 4-fold above the control cells, and subsequent insulin challenge induced only a 1.3-fold increase in transport. The inclusion of E64 with the insulin incubation had no additional effect on the glucose transport profile (compare 9EI with 9I). Similar results were obtained after an 18-h incubation with insulin alone or insulin plus E64.

The data presented in Fig. 2 demonstrate that desensitization of the different signaling pathways modulated by insulin respond in different ways to treatment of the cells with E64.

The findings presented above with lipogenesis and glycogen synthesis are consistent with the interpretation that β'-mediating the insulin resistance in 3T3-L1 adipocytes. When the fragment is present, insulin resistance is demonstrated. When the production of the fragment is inhibited, insulin resistance is not demonstrated. However, earlier reports had demonstrated that IRS-1 is down-regulated by insulin treatment of 3T3-L1 adipocytes (37). As an early coupling protein in the insulin signal transduction pathway, down-regulation of IRS-1 could inhibit the propagation of signal down the insulin-induced cascade, and lead to insulin resistance. It is also possible that E64 might inhibit the down-regulation of IRS-1. To test this, cytosol was isolated from adipocytes preincubated in the presence of insulin and/or E64. Following SDS-PAGE and transfer to nitrocellulose, the transfer was incubated with antibody directed against IRS-1. The immunoblot results are shown in Fig. 3A, and quantitation of the blot is presented in Fig. 3B. In the cytosol from control cells (lanes 1 and 2), IRS-1 was detected at a mass of 160 kDa, consistent with previous reports (37). Treatment of the adipocytes for 5 h with insulin resulted in a 50% decrease in immunodetectable IRS-1 (lanes 3 and 4), and exposure of the intact cells to insulin for 18 h further down-regulated the level of IRS-1 to approximately 20% of control levels (lanes 7 and 8). The addition of E64 to the insulin incubation had no effect on the level of expression of IRS-1. After 5 h of incubation with both insulin and E64 (lanes 5 and 6), IRS-1 was down-regulated to the same degree demonstrated in the presence of insulin alone, and after 18 h of incubation with both insulin and E64 (lanes 9 and 10), no detectable change in the level of IRS-1 could be observed compared to the level of IRS-1 seen with insulin alone. Therefore, in contrast to the effect of E64 on the production of β'-, E64 had

Fig. 2. Insulin-induced lipogenesis, glycogen synthesis, and glucose uptake in basal and insulin-resistant 3T3-L1 adipocytes: effects of E64 on insulin resistance. Panel A, effect of E64 on insulin-induced lipogenesis in cells chronically pretreated with insulin. 3T3-L1 adipocytes were pretreated with 1.7 μM insulin alone for 0 (C), 9 (9I), or 18 h (18I), or with insulin plus 100 μM E64 for 9 (9EI) or 18 h (18EI). Following exhaustive washing to remove any residual insulin, the cells were further stimulated with 0 nm insulin (black bars), 0.05 nm insulin (gray bars), or 10 nm insulin (cross-hatched bars) before quantitating lipogenesis as described under "Experimental Procedures." Under basal conditions in the control cells, glucose incorporation into lipids denoted as 1250 "cpm glucose incorporated into lipid" is equivalent to a rate of 13.5 nmol/min/10⁶ cells. Panel B, effect of E64 on insulin-induced glycogen synthesis in cells chronically pretreated with insulin. 3T3-L1 adipocytes were pretreated with 1.7 μM insulin alone for 0 (C), 9 (9I), or 18 h (18I), or with insulin plus 100 μM E64 for 9 (9EI) or 18 h (18EI). Following exhaustive washing to remove any residual insulin, the cells were further stimulated with 0 nm insulin (black bars), 5 nm insulin (gray bars), or 25 nm insulin (cross-hatched bars) before quantitating glycogen synthesis as described under "Experimental Procedures." Panel C, effect of E64 on insulin-induced glucose uptake in cells chronically pretreated with insulin. 3T3-L1 adipocytes were pretreated with 1.7 μM insulin alone for 0 (C), 9 (9I), or 18 h (18I), or with insulin plus 100 μM E64 for 9 (9EI) or 18 h (18EI). Following exhaustive washing to remove any residual insulin, the cells were further stimulated with 0 nm insulin (black bars), 2.5 nm insulin (gray bars), or 25 nm insulin (cross-hatched bars) before quantitating glucose uptake as described under "Experimental Procedures."
no effect on the insulin-induced down-regulation of IRS-1. Therefore, the insulin resistance induced by hyperinsulinemia in 3T3-L1 adipocytes is not mediated by a decrease in the level of IRS-1.

It has been demonstrated that the insulin-induced autophosphorylation of the insulin receptor is reduced in insulin-resistant tissues from normal individuals (29–32). We proceeded to ascertain if, under in vitro conditions, β’ was able to inhibit the insulin-induced autophosphorylation of the intact β subunit of the insulin receptor. Intact insulin receptor was partially purified from untreated, insulin-free 3T3-L1 adipocytes. β’ was partially purified from the cytosol of 3T3-L1 adipocytes that had been treated for 18 h with 1.7 µM insulin. A constant amount of intact receptor (732 kDa) was utilized in this experiment (0–140 m). The relative amounts of intact receptor and β’ utilized in this experiment were determined so that the amount of intact β subunit present in 7 µl of detergent extract was equivalent, by immunoblot analysis, to the amount of β’ present in 75 µl of purified cytosol. After mixing and incubation with or without insulin and [γ-32P]ATP, the samples were subjected to SDS-PAGE and radiometric scanning to quantitate the radioactivity associated with the insulin receptor β subunit at 92 kDa, as described under “Experimental Procedures.” Panel A, autoradiography of the intact β subunit as a function of the relative ratio of β’ to β subunit. The value of β’/β was quantitated as described under “Experimental Procedures.” Black bars, incubation in the presence of insulin. Gray bars, incubation in the absence of insulin. Panel B, fold stimulation of insulin receptor autophosphorylation as a function of the ratio of β’ to β. Black bars, stimulation of insulin receptor autophosphorylation was calculated as (dpm incorporated with insulin)/(dpm incorporated without insulin). A value of unity indicates no insulin-stimulated autophosphorylation of the insulin receptor β subunit.

Increased, insulin-induced stimulation of incorporation was inhibited. Interpolation of the data shown in Fig. 4B indicates that the insulin-induced phosphorylation of β subunit was totally inhibited at a β’/β ratio of approximately 1.

Interestingly, [32P]phosphate was also added to a protein band migrating with a mass of 61 kDa, corresponding to β’. Quantitation of the radioactivity in the β’ 61-kDa band is shown in Fig. 5. Phosphorylation of β’ occurred to an equal extent in the absence or presence of stimulation with insulin, and the level of incorporation increased as a linear function of the amount of added β’. Phosphorylation of β’ also occurred in the absence of intact insulin receptor (data not shown, manuscript in preparation). Therefore, from the data presented in Figs. 4 and 5, under in vitro conditions, β’ stoichiometrically inhibited the insulin-induced phosphorylation of the β subunit of the intact insulin receptor. In addition, β’ was itself phosphorylated.

The data presented in Figs. 1 and 2 suggest that E64 inhibits cellular insulin resistance by inhibiting the production of the
fragment of the insulin receptor β subunit, β'. To assess the effect of E64 on the insulin-resistant state manifest in the complex system of a whole animal, we utilized the obese, insulin-resistant Zucker fatty rat. This rodent model has been utilized as a model for insulin resistance and Type 2 non-insulin-dependent diabetes mellitus (42–44). The animals, both obese and age-matched lean Zucker rats, were injected with E64 on a daily basis for 3 months. The protocol was initiated at an age when the insulin-resistant state (determined by serum triglyceride levels) was fully expressed (42, 45). Serum insulin, glucose, and triglycerides were measured weekly. Consistent with earlier reports, we found the obese rats to be normoglycemic (185 mg/dl in both the fatty and lean rats) but hyperinsulinemic (40 microunits/ml in the obese animals, and 7 microunits/ml in the lean animals), compared to the non-obese animals. No statistically significant changes in serum glucose and insulin levels were noted over the course of the protocol in comparing the treated and control animals (data not shown). However, the obese animals had elevated blood levels of triglycerides compared to the lean rats. Lean rats injected with E64 demonstrated no change in blood levels of triglycerides compared to saline-injected controls (data not shown). Weights of neither the obese nor the lean rats was changed by E64 compared to the saline controls (data not shown). The effect of daily injection of E64 on serum triglyceride levels is shown in Fig. 6. In the 2-week period preceding the beginning of the E64 administration, the blood levels of triglycerides were comparable in both the test and control group of animals. Over the next 3 months of the protocol, the blood levels of triglycerides in the E64 group were maintained at the pretreatment levels. However, the obese rats injected with saline demonstrated a steady increase in blood triglyceride levels over the 3-month protocol. Linear regression analysis of the triglyceride levels in E64-treated rats demonstrated an increase in blood levels of only 0.62 mg/dl/day, while the saline control animals increased blood triglyceride levels at a rate of 7.34 mg/dl/day. Therefore, E64 inhibited the rate of increase in blood triglycerides by a factor of 11.8. While the injection of E64 did not reverse the insulin-resistant state manifest by the rodents at the onset of the protocol, it prevented the worsening of the insulin-resistant state.

To determine the effect of E64 treatment on the level of intact insulin receptor and β' in the tissues of the rodents, liver, skeletal muscle, and adipose tissue were excised from both lean and obese animals, extracted with detergent-containing buffers, and the extracts were preabsorbed with normal rabbit IgG and then immunoprecipitated with anti-P5 antibody prior to immunoblot analysis with anti-P5 antibody. The intensity of the bands at 92 kDa (intact β subunit) were determined, and the results are presented in Fig. 7. All samples were analyzed simultaneously. Therefore, direct comparisons between samples are possible. It should also be noted that adipose tissue has a relatively high receptor level. Consequently, in order to maintain all of the samples within the linear range of detection, 0.1 mg of adipose total protein was subjected to immunoprecipitation compared to 1 mg of liver or muscle protein. In the lean rats (L), E64 treatment had no effect on the level of intact insulin receptor β subunit in any tissue. A comparison of the level of the β subunit in the untreated lean animals (L/Saline) and the untreated obese (F/Saline) rats indicated that in all three tissues, β subunit levels are substantially down regulated in the obese rats. Total β subunit level is decreased by 4-fold in the untreated liver of the obese rats, and comparable decreases are demonstrated in muscle and adipose tissue. E64 treatment of the obese rats dramatically reversed the down-regulation of the levels of β subunit (compare F/Saline with F/E64). With all three tissues, E64 treatment of the animals resulted in increased levels of intact β subunit such that the β subunit levels in the fatty rats treated with E64 were comparable to the levels of β subunit present in the lean rats.

Thus, the data presented in Fig. 7 are consistent with the interpretation that E64 inhibits the insulin-induced proteolysis of the intact β subunit of the insulin receptor, thereby elevating the level of receptor in the tissues of these animals, improving their insulin resistance.

The effect of E64 treatment on the level of β' in the tissues of the obese rats is shown in Fig. 8. As described above for the quantitation of the intact β subunit, 0.1 mg of adipose protein was subjected to immunoprecipitation compared to 1 mg of liver, muscle, kidney, or spleen protein. In the obese rats, E64 treatment resulted in decreased levels of β' in muscle, adipose tissue, and kidney by factors of 1.9, 2.2, and 1.4, respectively. Spleen, a tissue not normally viewed as an insulin target tissue, demonstrated no E64-induced change in β'. Paradoxically, liver from E64-treated rats demonstrated an elevated level of β'. We currently have no explanation for this finding. A low level of β' could be detected in the tissues of the lean rats, and E64 treatment had no effect on the level of expression of β' in the tissues of these animals (data not shown).

**DISCUSSION**

We had previously demonstrated that in fibroblasts, chronic insulin treatment induces the proteolytic release of a fragment...
of the insulin receptor in tissues from Zucker rats. Upon completion of the protocol described in the text and Fig. 6, tissue was excised from the animals, extracted into detergent-containing buffer, and the level of the intact \( \beta \) subunit of the insulin receptor was quantitated, as described under "Experimental Procedures." In each immunoprecipitation, 1 mg of extracted protein from liver and muscle was utilized, and 0.1 mg of extracted protein from adipose tissue was utilized. F/Saline, fatty Zucker rats injected with saline alone; F/E64, fatty Zucker rats injected with E64; L/Saline, lean Zucker rats injected with saline alone; L/E64, lean Zucker rats injected with E64. Black bars, liver; gray bars, muscle; cross-hatched bars, adipose tissue.

![Figure 7](image)

**Fig. 7.** The effect of E64 on the level of the \( \beta \) subunit of the insulin receptor in tissues from Zucker rats. Upon completion of the protocol described in the text and Fig. 6, tissue was excised from the animals and extracted into detergent-free buffer, and the level of the \( \beta \) subunit of the insulin receptor was quantitated, as described under "Experimental Procedures." In each immunoprecipitation, 1 mg of extracted protein from liver, muscle, kidney, and spleen was utilized, and 0.1 mg of extracted protein from adipose tissue was utilized. When \( \beta' \) is present, the cells are insulin-resistant, but when \( \beta' \) is reduced or absent, the cells are insulin-sensitive. The same correlation is demonstrated with an alternate index of insulin sensitivity, glycogen synthesis. Insulin-induced glycogen synthesis is absent from the 3T3-L1 adipocytes when the cells are pretreated for 5–18 h with insulin alone, but under conditions that inhibit the production of \( \beta' \) (simultaneous treatment with E64), insulin sensitivity is retained. There is, however, a significant difference between the effect of E64 on lipogenesis versus glycogen synthesis. With lipogenesis, E64 maintains the basal level of lipogenesis at levels comparable to the level seen in the control cells. In contrast, with glycogen synthesis, cells treated with both E64 and insulin demonstrate an elevated rate of basal incorporation similar to that seen in cells treated with insulin alone. An even greater discrepancy exists in the effects of E64 on the insulin-induced desensitization of glucose transport. E64 had no effect in these cells on the recovery of desensitized glucose uptake. The basis for these differences is unknown, but possibly reflects differences in the insulin-induced signaling pathways for lipogenesis, glycogen synthesis, and glucose transport. Subtle differences in the pathways for lipogenesis and glycogen synthesis may exist, but more dramatic differences between the signaling pathway leading to glucose transport versus lipogenesis are apparent.

![Figure 8](image)

**Fig. 8.** The effect of E64 on the level of \( \beta' \) production in tissues from obese Zucker rats. Upon completion of the protocol described in the text and Fig. 6, tissue was excised from the animals and extracted into detergent-free buffer, and the level of \( \beta' \) was quantitated as described under "Experimental Procedures." In each immunoprecipitation, 1 mg of extracted protein from liver, muscle, kidney, and spleen was utilized, and 0.1 mg of extracted protein from adipose tissue was utilized.

A major problem with the interpretation of the data of Figs. 4 and 5 is that the thiol protease inhibitor E64 inhibited insulin action in fibroblasts at concentrations of 2.5 μM. E64 is also able to inhibit tyrosine kinase activity in fibroblasts at concentrations of 10 μM. Therefore, it is possible that the effects of E64 on insulin action may be due to the inhibition of tyrosine kinase activity. However, this is unlikely since E64 has no effect on the insulin-induced down-regulation of IRS-1. Under conditions of concomitant insulin and E64 treatment, where insulin responsiveness is maintained, the level of IRS-1 is down-regulated to the same degree as in the presence of insulin alone. However, the data of Fig. 3 clearly demonstrate that E64 has no effect on the insulin-induced down-regulation of IRS-1. Under conditions of concomitant insulin and E64 treatment, where insulin responsiveness is maintained, the level of IRS-1 is down-regulated to the same degree as in the presence of insulin alone. Therefore, down-regulation of IRS-1 does not contribute to the insulin-induced insulin resistance of 3T3-L1 adipocytes when utilizing lipogenesis and glycogen synthesis as indices of insulin action. However, glucose transport, like IRS-1 levels, are not affected by the E64, suggesting that IRS-1 levels become limiting in the induction of glucose transport in the desensitized cells.

A mechanism to support the correlation between E64, \( \beta' \), and insulin resistance is provided by the data of Figs. 4 and 5. Coincubation of intact insulin receptor with partially purified \( \beta' \) results in the inhibition of the insulin-induced autophosphorylation of the intact \( \beta \) subunit. When the ratio of \( \beta \) subunit to \( \beta' \) is unity, total inhibition of phosphorylation is demonstrated. In the intact 3T3-L1 adipocytes, total insulin resistance was found after 9 h of incubation with insulin. We have previously demonstrated that after 5–6 h of incubation of these cells with insulin, the receptor level declines by approximately 50% (28). As the receptor down-regulates, \( \beta' \) is generated, such that by 5–6 h after insulin addition, the concentration of intact \( \beta \) subunit in the cellular membranes should be approximately equal to the concentration of \( \beta' \) in the cytosol. Therefore, sufficient quantities of \( \beta' \) are generated in the 3T3-L1 adipocytes by 9 h to account for the complete insulin resistance demonstrated in Fig. 2.
preparation, $\beta'$ is not the only protein in the preparation (data not shown). Therefore, it is possible that $\beta'$ itself is not inhibiting the autophosphorylation of the insulin receptor but that another factor in the preparation, which is induced by insulin treatment and inhibited by E64 treatment, could be affecting receptor autophosphorylation. However, our preliminary data indicate that cystosol isolated from control cells or cells incubated with both insulin and E64 does not have any effect on insulin receptor autophosphorylation (data not shown). Resolution of this question will be possible when we have purified $\beta'$ to homogeneity. Our current efforts are directed in this area.

$\beta'$ is itself phosphorylated, but in an insulin-independent manner. This finding is consistent with results generated with deletion mutants of the insulin receptor. In these studies, insulin receptor cDNA sequences coding for the extracellular domain of the receptor were deleted, resulting in the expression of a membrane-anchored (46) or fully soluble (47–49) cytoplasmic domain of the insulin receptor. This protein was found to be a constitutively active tyrosine kinase, contributing to the conclusion that the insulin binding domain of the insulin receptor acts in a negative regulatory manner, inhibiting the tyrosine kinase activity of the $\beta$ subunit (46). Studies are currently under way in our laboratory to ascertain if $\beta'$ has tyrosine kinase activity. If it does have this activity, it would present a potential new mechanism of insulin resistance; $\beta'$ not only inhibits the insulin receptor autophosphorylation, but if $\beta'$ has tyrosine kinase activity, it may inappropriately phosphorylate substrates, resulting in aberrant insulin signaling.

The studies cited above on the expression of the cytoplasmic domain of the insulin receptor were apparently not extended to determine the effect of this domain on the signal transduction capacity of the endogenous insulin receptor. However, studies of this nature have been performed with the $\alpha$-adrenergic receptor (41). The third cytoplasmic loop of the $\alpha_{1B}$ adrenergic receptor has been expressed as a soluble protein in cells along with the parent $\alpha_{1B}$ adrenergic receptor. The expression of the cytoplasmic domain has been found to inhibit the ability of the parent receptor to activate phospholipase C. Therefore, this study on the $\alpha$-adrenergic receptor forms a precedent for our finding that a fragment of the cytosolic domain of the insulin receptor inhibits receptor function. However, in this study on the adrenergic receptor, the cytosolic fragment of the receptor was expressed as a result of transfection with modified DNA; the fragment is not a protein normally expressed in the wild-type cell. In the study we report here, the fragment of the insulin receptor is expressed in wild-type cells, in response to "pathological" concentrations of insulin, resulting in cellular insulin resistance.

To determine the effect of E64 on insulin resistance under in vivo conditions, Zucker fatty rats were injected with E64 on a daily basis. As shown in Fig. 6, E64 has an immediate effect on serum triglyceride levels, maintaining the triglyceride levels in the treated rats at pretreatment levels. This was in marked contrast to the saline-treated fatty rats, where the serum triglyceride levels continued to increase. Therefore, injection of E64 prevented the worsening of hyperlipidemia in this animal model. The data presented in Fig. 7 demonstrate that concomitant with an E64-induced improvement in the insulin resistance of the obese rats, the level of the $\beta$ subunit in insulin target tissues recovered from a down-regulated state. These data substantiate the interpretation that E64 prevents the insulin-induced degradation of the insulin receptor. Fig. 8 demonstrates the effect of the E64 protocol on $\beta'$ production in tissues from obese Zucker rats. In muscle, adipose tissue, and kidney, E64 decreased $\beta'$ levels approximately 2-fold. These data are consistent with the view that E64 blocks $\beta'$ production, leading to the observed improvement of the insulin-resistant state shown in Fig. 6.

It is important to note that this animal protocol was initiated when the insulin-resistant state was fully manifest in these rats. Additional studies are in progress to ascertain if hyperlipidemia can be prevented in the Zucker fatty rats by administration of E64 to the animals earlier in their lives, before the onset of florid insulin resistance. A significant drawback to the use of Zucker fatty rats to study insulin resistance is that, unlike insulin resistance in humans, the Zucker fatty rats are normoglycemic. Therefore, glycemic control cannot be utilized as an index of the degree of insulin resistance. Future animal studies on the use of E64 will include other animal models of insulin resistance, where changes in blood glucose and insulin can be utilized as indices of the effectiveness of E64 in decreasing insulin resistance.

In summary, this report demonstrates a causal link between the production of $\beta'$, a cytosolic fragment of the insulin receptor, and the generation of insulin resistance. Insulin treatment of cultured adipocytes results in a time-dependent loss of intact $\beta$ subunit from the cellular membranes, and a concomitant generation of $\beta'$ in the cell cytosol, in which the production of $\beta'$ is inhibited by the thiol protease inhibitor E64. While insulin treatment of the adipocytes also results in insulin resistance, as measured by the loss of insulin-stimulated lipogenesis, glycerogen synthesis, and glucose uptake, concurrent treatment of the adipocytes with insulin and E64 inhibits the generation of the insulin-resistant components of IRS-1 in 3T3-L1 adipocytes. In vitro experiments, partially purified $\beta'$ inhibits the insulin-induced autophosphorylation of the $\beta$ subunit of the intact insulin receptor, such that complete inhibition of insulin-induced autophosphorylation is achieved when the molar ratio of $\beta'$ to $\beta$ subunit is unity. This suggests that $\beta'$ induces insulin resistance in intact cells by inhibiting the autophosphorylation of the insulin receptor. Finally, in intact animals, E64 improves the insulin-resistant state of Zucker fatty rats, lowering the blood levels of triglycerides in this rat model of insulin resistance. In this animal model, administration of E64 both increases the level of intact $\beta$ subunit and decreases the level of $\beta'$ in the animal tissues, when compared to the corresponding tissues from saline-treated animals. Therefore, these data indicate that insulin resistance is mediated by a cytosolic fragment of the insulin receptor, $\beta'$, which is generated by hyperinsulinemia.

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