7-Hydroxystaurosporine (UCN-01) Inhibition of Akt Thr<sup>308</sup> but not Ser<sup>473</sup> Phosphorylation: A Basis for Decreased Insulin-Stimulated Glucose Transport

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ABSTRACT
7-Hydroxystaurosporine (UCN-01) infused for 72 hours by continuous i.v. infusion induced insulin resistance during phase I clinical trials. To understand the mechanism for this effect, we examined the effect of UCN-01 on insulin-stimulated glucose transport activity with 3-O-methylglucose in isolated rat adipose cells. UCN-01 inhibits glucose transport activity in a dose-dependent manner at all insulin concentrations. At the clinically relevant concentration of 0.25 μmol/L UCN-01, glucose transport is inhibited 66, 29, and 26% at insulin concentrations of 10, 50, and 100,000 (100K) microunits/mL respectively, thus shifting the dose-response curve to the right. Increasing concentrations of UCN-01 up to 2.5 μmol/L progressively shift the insulin dose-response curve even further. As Akt is known to mediate in part action initiated at the insulin receptor, we also studied the effect of UCN-01 on Akt activation in whole-cell homogenates of these cells. Decreased glucose transport activity directly parallels decreased Akt Thr<sup>308</sup> phosphorylation in both an insulin and UCN-01 dose-dependent manner, whereas Akt Ser<sup>473</sup> phosphorylation is inhibited only at the lowest insulin concentration, and then, only modestly. UCN-01 also inhibits insulin-induced Thr<sup>308</sup> but not Ser<sup>473</sup> phosphorylation of Akt associated with the plasma membranes and low-density microsomes and inhibits translocation of GLUT4 from low-density microsomes to plasma membranes as expected from the glucose transport activity measurements. These data suggest that UCN-01 induces clinical insulin resistance by blocking Akt activation and subsequent GLUT4 translocation in response to insulin, and this effect appears to occur by inhibiting Thr<sup>308</sup> phosphorylation even in the face of almost completely unaffected Ser<sup>473</sup> phosphorylation.

INTRODUCTION
Novel cancer therapies are in development, which target the specific molecular events driving cancer cell proliferation and metastasis. Several protein kinases have been identified as key regulators of these processes, and therefore, protein kinase inhibitors are of great interest in cancer therapeutics. 7-Hydroxystaurosporine (UCN-01) is one such compound that occurs naturally in a soil bacterium. It was originally identified as a potent (<100 nM) inhibitor of calcium-dependent protein kinase C (PKC)-α, -β, and -γ isoforms. Calcium independent PKC-δ and ε were inhibited only at higher concentrations (>500 nM) of UCN-01. PKC-ζ was not inhibited by UCN-01 (1, 2). Subsequent studies revealed that it also potently inhibits the DNA damage response regulatory kinases chk1 and possibly chk2 (3–5) and phosphatidylinositol-dependent kinase 1 (PDK-1; ref. 6). At higher concentrations (>1 μM/L), UCN-01 inhibits many other kinases. UCN-01 was also found to have potent activity in the National Cancer Institute’s 60 human cancer cell line drug screen and to demonstrate antineoplastic activity in a number of pre clinical animal models (7, 8). On the basis of its novel antineoplastic properties, initial phase I clinical trials were completed (9). Although a safe dose and treatment schedule could be defined, dose-limiting toxicities included unexpected hyperglycemia, with frequent lesser degrees of hyperglycemia documented at lower doses. Initial studies suggested that the basis for this effect was peripheral tissue insulin resistance because circulating insulin and C-peptide levels increased (9).

Acute insulin treatment stimulates glucose transport in adipose and muscle cells largely through the translocation of GLUT4 from an intracellular compartment to the plasma membrane (10–13). Upon insulin binding, the tyrosine kinase activity of the insulin receptor is activated, which in turn initiates a complex signaling cascade (14). The downstream activation of a Wortmannin-sensitive phosphatidylinositol 3′-kinase appears to be essential for the metabolic effects of insulin, including GLUT4 translocation and increased glucose uptake (14–17).

Akt/PKB is a downstream Ser/Thr kinase activated by phosphatidylinositol 3′-kinase. Akt/PKB interacts with the lipid products of phosphatidylinositol 3′-kinase, but it is not yet fully clear how the insulin signal is relayed to the glucose transporters (16, 18, 19). Full activation of Akt by insulin appears to require hierarchical phosphorylation of two residues, Thr<sup>308</sup> and Ser<sup>473</sup>,
originally proposed to be because of the action of PDK-1 and PDK-2, respectively (20–24). Recent reports have suggested that overexpression of myristoylated Akt increases GLUT4 translocation and glucose transport activity to levels similar to or greater than those achieved with insulin in rat adipose cells (19), L6 muscle cells (25, 26), and 3T3-L1 adipocytes (12, 26, 27). The actual identity of the molecule responsible for PDK-2 activity remains a subject of active investigation.

The recent elucidation of PDK-1 as a potently affected target of UCN-01 led us to hypothesize that clinical insulin resistance in patients receiving UCN-01 might be the direct effect of UCN-01 on glucose uptake mechanisms. In the present study, we examined the effects of UCN-01 on insulin-induced GLUT4 translocation and glucose transport activity in rat adipose cells. Because UCN-01 is a PDK-1 inhibitor, we also examined the phosphorylation state and activity of Akt. We present data consistent with the hypothesis that UCN-01 induces insulin resistance by blocking Akt activation and subsequent GLUT4 translocation in response to insulin, and this effect appears to occur by inhibiting Akt Thr308 phosphorylation, even in the face of almost completely unaffected Akt Ser473 phosphorylation.

MATERIALS AND METHODS

Isolated Rat Adipose Cell Preparation. Isolated adipose cells were prepared by collagenase digestion of epididymal fat pads from 170 to 200-g male CD rats (Charles River Breeding Laboratories, Boston, MA) as described previously (28).

Glucose Transport Activity. Glucose transport activity was measured in intact adipocytes by the 3-O-methylglucose uptake technique as described previously (28). To examine the inhibition of insulin-induced glucose transport by UCN-01, adipocytes were preincubated with various concentrations of UCN-01 for 30 minutes before measurement of methyl-3-glucose uptake as described previously (28).

Incubation with Insulin and Subcellular Fractionation. Before fractionation, isolated adipose cells were pretreated with UCN-01 at various concentrations for 30 minutes and then incubated in the absence or presence of insulin (10, 50 and 100,000 (100K) microunits/mL) at 37°C for 2 to 30 minutes. The cells were then homogenized in 10 mmol/L Tris (pH 7.4), 255 mmol/L sucrose, and 1 mmol/L EDTA containing protease and phosphatase inhibitors (Sigma) and fractionated by differential ultracentrifugation as described (28). Three fractions were recovered: plasma membranes, high-density microsomes enriched in endoplasmic reticulum, and low-density microsomes enriched in Golgi vesicles. These subcellular membrane fractions have been characterized elsewhere (28). Part of the homogenate was subjected to a single ultracentrifugation at 200,000 × g for 20 minutes to obtain a cytosolic fraction (supernatant) and total cellular membranes (pellet). Membrane pellets were resuspended in homogenization buffer containing protease inhibitors, and the protein content was determined by the BCA assay (Pierce, Rockford, IL).

Immunoblotting. Cytosolic and membrane proteins were subjected to denaturing electrophoresis with 4 to 20% polyacrylamide gels (Invitrogen, Gaithersburg, MD). The content of the gels was transferred by electroblotting onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked with 5% Blotto, nonfat dry milk (Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection of Akt, phospho-Akt, glycogen synthase kinase (GSK-3; Cell Signaling Technology, Beverly, MA), and GLUT4 was carried out with a rabbit polyclonal antiserum prepared against a COOH-terminal peptide (8107P; kindly provided by Hoffman-La Roche, Nutley, NJ). After 2 hours of incubation in primary antibody, the blots were washed with 1× PBS with Tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ) for 1 hour. The blots were washed as above, and the immunolabeled bands were visualized with ECL plus chemiluminescent kit (Amersham). The densitometric analysis was carried out using ImageQuant software (Molecular Devices, Sunnyvale, CA).

Akt Kinase Assay. Recombinant active Akt assay kit was purchased from Cell Signaling Technology. The assay was performed according to the manufacturer’s instructions with the GSK-3 fusion protein as substrate peptide.

RESULTS

UCN-01 Inhibits Insulin-induced Glucose Transport Activity in Rat Adipose Cells. With no available data regarding the inhibitory effects of UCN-01 on glucose transport, we started off with very high (10 and 50 μmol/L) drug concentrations, which made the cells leaky (data not shown). After several trials, we decided to expose freshly isolated adipose cells to various concentrations of UCN-01 for 30 minutes and subsequently stimulated them without (basal) or with insulin for an additional 30 minutes. Isolated rat adipose cells typically respond to insulin over the range of 0 to 100 microunits/mL or 0 to 670 pmol/L, with a half-maximal response at 10 to 20 microunits/mL (67 to 134 pmol/L). Because we postulated that the effect of UCN-01 would be a reduced sensitivity to insulin, i.e., a right-shifted insulin dose-response curve, we designed the experimental conditions to cover a maximum range of reduced activity over the broad range of insulin binding to its...
receptor \(K_a \approx 1 \text{nmol/L}\). As demonstrated in Fig. 1, all cells exposed to UCN-01 (0.25 to 2.5 \text{mmol/L}) show a dose-dependent inhibition of insulin-stimulated 3-O-methylglucose transport. At 0.25 \text{mmol/L} UCN-01, glucose transport activity is inhibited 66, 29, and 26\% at insulin concentrations of 10, 50, and 100,000 (100K) microunits/mL, thus shifting the insulin dose-response curve to the right. Increasing concentrations of UCN-01 up to 1.0 \text{mmol/L} progressively shift the insulin dose-response curve even further, whereas 2.5 \text{mmol/L} UCN-01 actually inhibits maximally stimulated glucose transport activity.

**Effect of UCN-01 on Akt Phosphorylation in Total Homogenates.** Recent studies have collectively suggested that activation of Akt is a mediator of glucose transport by insulin stimulation (29–31). Full activation of Akt by insulin appears to require phosphorylation of Thr\(^{308}\) and Ser\(^{473}\) by PDK-1 and the putative PDK-2, respectively. Because UCN-01 has recently been shown to potently inhibit PDK-1 (6, 32, 33), we thought the inhibition of insulin-stimulated glucose transport may be due at least in part to inhibition of Thr\(^{308}\) phosphorylation, resulting in failure to activate Akt. To validate this hypothesis, we examined Akt phosphorylation state in total adipose cell homogenates. At 10 microunits/mL insulin (Fig. 2A) and 50 microunits/mL insulin (Fig. 2B), the lowest dose (0.25 \text{mmol/L}) of UCN-01 almost completely inhibits Thr\(^{308}\) phosphorylation. Akt Thr\(^{308}\) phosphorylation in response to maximum insulin 10,000,000 (100K) microunits/mL is almost completely inhibited by 2.5 \text{mmol/L} UCN-01 (Fig. 2C). Interestingly, Ser\(^{473}\) phosphorylation is not affected by 0.25 \text{mmol/L} UCN-01 at 10 microunits/mL insulin by any concentrations of UCN-01 at 50 and 100K microunits/mL insulin (Fig. 2). The relative densities of Thr\(^{308}\) and Ser\(^{473}\) measured against total Akt are shown as ratios below each panel and clearly indicate the differences in Thr\(^{308}\) and Ser\(^{473}\) phosphorylation due to UCN-01. These results suggest that UCN-01 can clearly distinguish between the kinase activities directed at Akt Thr\(^{308}\) (presumably PDK-1) and Akt Ser\(^{473}\).

**UCN-01 Inhibits Membrane Translocation of Akt Thr\(^{308}\)-phosphorylated Akt.** The two primary sites of insulin-dependent glucose disposal are muscle and fat where the primary insulin-sensitive glucose transporter GLUT4 is synthesized and redistributed from intracellular storage vesicles to the plasma membrane after insulin stimulation. Activated Akt plays a primary role in directing GLUT4 vesicles to the plasma membrane and thus promotes glucose transport. The inhibition of Akt activity by interfering antibodies, substrate peptides, or synthesis of dominant negative Akt partially blocks insulin-stimulated GLUT4 translocation (12, 19, 25–27). Therefore, we studied the effects of UCN-01 on Akt activity and the subcellular distribution of Akt and GLUT4. Cells were pretreated with UCN-01 and then stimulated with insulin for 2, 6, 10, and 20 minutes. As demonstrated in Fig. 2D, Akt phosphorylation in response to insulin is observed within 2 minutes in both the cytosolic and membrane fractions, suggesting that insulin stimulation is immediate and sustained. Interestingly, UCN-01 blocks insulin-induced Thr\(^{308}\) Akt phosphorylation in both plasma membranes and low-density microsomes but not Ser\(^{473}\) phosphorylation at all of the time points (Fig. 3, A and B). In plasma membranes, Akt protein only marginally increases, if it changes at all, in response to insulin, but phospho-Thr\(^{308}\)-specific activity actually decreases, and phospho-Ser\(^{473}\)-specific activity stays elevated and constant (Fig. 3A). However, in the absence of UCN-01, we observe with insulin treatment a progressive increase in Akt protein in the low-density microsome fraction, with constantly elevated phospho-Thr\(^{308}\)-specific activity and decreasing phospho-Ser\(^{473}\)-specific activity (Fig. 3B). The presence of UCN-01 is associated with increased

*Fig. 2 Immunoblots showing inhibition of Akt phosphorylation by UCN-01. Protein extracted from adipose cells, which were pretreated with UCN-01 and then stimulated with insulin (INS) as indicated (A–C). UCN-01 completely inhibited INS-induced Thr\(^{308}\) phosphorylation of Akt at lower concentrations (A and B). Ser\(^{473}\) phosphorylation was not inhibited. Thr\(^{308}\) phosphorylation was observed within 2 minutes: both in cytosolic and total membrane fractions in the presence of INS (D). The numbers at the bottom of each panel indicate the relative density values for Thr\(^{308}\) and Ser\(^{473}\), respectively, for A, B, and C.*

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**A**

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Akt protein in plasma membranes and prevention of the increase in Akt protein in low-density microsomes in response to insulin. To verify the status of GLUT4, we examined the low-density microsomes and plasma membranes fractions for GLUT4 with rabbit antiserum. Insulin induces a progressive translocation of GLUT4 from low-density microsomes to plasma membranes for 10 minutes, and this is prevented by UCN-01 (Fig. 3). The most impressive change is observed at the end of 2 minutes, where the translocation of GLUT4 is completely blocked. We observe an ~50 to 70% decline in translocation at 6 and 10 minutes.

**Akt Kinase Activity Is Inhibited by UCN-01.** Several earlier experiments have shown that phosphorylation of both Thr<sup>308</sup> and Ser<sup>473</sup> of Akt is required for complete Akt activation. In the data provided above and Fig. 4A, we have established that UCN-01 can completely inhibit Akt Thr<sup>308</sup> phosphorylation without significantly affecting on Akt Ser<sup>473</sup> phosphorylation (Fig. 4A). To confirm that inhibition of Thr<sup>308</sup> phosphorylation of Akt was
associated with the decrease in Akt kinase activity, we measured activity by immunoprecipitating Akt from the cytosolic fraction. We used GS3-3 fusion protein as substrate for the kinase assay as shown in Fig. 4B, and Akt kinase activity is completely inhibited to control levels at all time points, indicating that inhibition of Akt Thr308 phosphorylation is sufficient to result in the loss of Akt activity. As might be expected, we also observed that insulin-induced endogenous cellular GS3-3 phosphorylation is blocked by UCN-01 (data not shown).

DISCUSSION

In an effort to define the mechanism for clinically observed UCN-01–induced dose-limiting hyperglycemia, with evidence of insulin resistance (9), we have demonstrated in this study that UCN-01 inhibits in a dose-dependent manner insulin-stimulated (as low as 10 micromoles/mL) glucose transport in rat adipose cells. As Akt has been known to be an intermediate in insulin signaling, we went on to demonstrate that UCN-01 inhibits Thr308 but not Ser473 phosphorylation of Akt, a finding reported here for the first time, which is concordant with reduced Akt kinase activity after exposure to UCN-01. Inhibition of phosphorylation of Akt Thr308 in the plasma membrane and glucose transporter GLUT4 translocation to the plasma membrane by UCN-01 correlated with the inhibition of glucose transport, indicating a functionally important consequence of these molecular events. These findings suggest that this drug is exerting inhibitory effects on glucose transport and thus insulin resistance through its effects on the PDK-1/Akt–signaling pathway.

The Akt gene was originally identified as the cellular homologue of v-akt. The catalytically inactive Akt exists in the cytosol. By activation of phosphoryl-3-inositol 3'-kinase, phosphatidylinositol(3,4,5)P3, and phosphatidylinositol(3,4)P2 are synthesized at the plasma membrane, and Akt is recruited to the membrane. Akt is activated after phosphorylation at two key regulatory sites: Thr308 in the activation loop of the catalytic domain and Ser473 in the COOH-terminal domain. Phosphorylation of Akt at Thr308 is catalyzed by the ubiquitously expressed PDK-1, a previously described target of UCN-01 (6). The kinase responsible for phosphorylation of Akt at Ser473 has been called PDK-2, although the exact molecular identification of Ser473 kinases has been a matter of debate in the literature (34). Autophosphorylation by Akt itself is not ruled out (35). Integrin-linked kinase has also been associated with phosphorylation of Akt on Ser473 (24), which is contested by other laboratories (36, 37).

This study provides evidence that in rat adipose cells, whereas the PDK-1–related activity is sensitive to UCN-01, consistent with prior reports (6), the Ser473–directed activity is almost totally insensitive to UCN-01. Therefore, UCN-01 could be a valuable pharmacological tool in additionally exploring the regulation of insulin signaling in this tissue. Our experiments are consistent with the idea that insulin resistance due to UCN-01 may be attributable, in part, to the overall inhibition of Akt Thr308 phosphorylation, thus leading to a compromise of glucose uptake.

Stauroporine is a natural product, long recognized as a potent, nonspecific inhibitor of PKC (a Ca2+- and phospholipid-activated kinase). Subsequent studies revealed that UCN-01 shows a greater selectivity than stauroporine for PKC isoforms (α, β, and γ; refs. 2, 38). It is of interest that we have found that insulin-specific effects on atypical PKCs (ζ) in total cell lysates of rat adipose cells remain the same in the presence or absence of UCN-01 (data not shown). Two other potentially affected target classes have emerged, which are identified as the DNA damage-checkpoint related-kinases chk1 and possibly chk2 (4, 39). Sato et al. (6) found that UCN-01 directly suppresses upstream Akt kinase PDK-1 in both in vitro and in vivo assays. We observed that UCN-01 also significantly inhibited Thr308 phosphorylation at 100 nmol/L but required higher concentrations (1 μmol/L) to inhibit Ser473 in LNCaP, MCF-7, and MDA-435 cancer cell lines. A recent report suggests that the antitumor activities of UCN-01 in Kaposi’s sarcoma allograft model correlated well with its inhibition of Akt (40). In the present study, the changes in glucose transport revealed that exposure of cells to UCN-01 abolishes Thr308 but not Ser473 phosphorylation (Figs. 2–4), blocks GLUT4 translocation to the plasma membrane (Fig. 3C), and inhibits in vitro phosphorylation of Akt substrate GS3-3 in adipose cells (Fig. 4). We extend these findings by additionally demonstrating a functionally important consequence of PDK-1 target inhibition. Specifically, inhibition in rat adipose cells of the PDK-1 target Akt Thr308 appears to result in diminished Akt activity and diminished glucose transport activity after inhibiting GLUT4 translocation.

These findings are of interest not only because they provide a plausible explanation for clinical insulin resistance with hyperglycemia after exposure to UCN-01, but also because they raise the possibility that hyperglycemia might be a class-specific effect of Akt inhibition. Because Akt is being approached as a target of interest to several academic and commercial drug discovery efforts, caution to assess the effect of candidate drug molecules on insulin signaling would seem prudent in view of the results presented here.

Estimation of the change of glucose transport activity revealed that treatment of adipose cells with UCN-01 had a significant down-regulatory effect at 0.25 μmol/L UCN-01, a clinically relevant and achievable free concentration of UCN-01 (9). Specifically, glucose transport activity is inhibited 66, 29, and 26% at insulin concentrations of 10, 50, and 100,000 (100K) micromoles/mL, thus clearly shifting the dose-response curve to the right. Increasing concentrations of UCN-01 up to 2.5 μmol/L progressively shift the INS dose-response curve even further.

It is also clear that insulin stimulates a rapid but transient (maximum at 2 minutes) phosphorylation of Akt Thr308 in the plasma membrane fraction (Fig. 3A), a gradually increasing (over 10 minutes) level of Thr308-phosphorylated Akt protein in the low-density microsomes, accompanied by decreasing Ser473 phosphorylation (Fig. 3B) and a rapid, then constant (maximum at 2 minutes) phosphorylation of Thr308 in cytosolic Akt (Fig. 4A). These observations suggest a highly choreographed movement of Akt among subcellular compartments during its activation and signaling, including the possibility that the appearance of Akt in the low-density microsomes with its reduced phospho-Ser473–specific activity represents Akt deactivation. The simul-
taneous inhibition by UCN-01 of Akt Thr$^{308}$ phosphorylation and the subcellular redistribution of Akt from the plasma membranes to the low-density microsomes supports this concept. Substantial evidence suggests that Akt plays a role in directing GLUT4 vesicles to the plasma membrane, whether this is by direct association with GLUT4 (41, 42) or indirectly (43–45). Constitutively active Akt mimics insulin in eliciting high levels of GLUT4 translocation in adipocytes in the absence of insulin. However, the inhibition of Akt activity by interfering antibodies, substrate peptides, or synthesis of dominant negative Akt only partially blocks GLUT4 translocation to plasma membranes. Although in this report, the GLUT4 translocation was reduced by 70% at the end of 6 and 10 minutes (Fig. 3C) unlike 100% at the end of 2 minutes, it was sufficient to completely inhibit glucose transport activity (Fig. 1). This phenomenon once again suggests that Akt activity is essential for glucose transport. It was also observed by others that ceramide dramatically inhibits insulin-induced glucose transport activity and this correlates with a 60% reduction Akt activity (25). In contrast, several studies found no correlation between Akt and glucose transport, e.g., in adipocytes stably transfected with dominant negative Akt: these studies showed that an 80% reduction in endogenous Akt activity does not impair insulin-stimulated glucose transport activity (46). The authors attributed this phenomenon to the residual Akt activity. However, in our experiments, all of the samples treated with UCN-01 retained Ser$^{473}$ phosphorylation but lost Thr$^{308}$ phosphorylation, Akt kinase activity, and glucose transport activity. Additional research is required to clarify these different observations. Contrary to our findings, Hernandez et al. (31), with ML-9, a chemical inhibitor of Akt activity, showed that it inhibits insulin stimulated Ser$^{473}$ phosphorylation but not Thr$^{308}$ phosphorylation. They claimed that ML-9 treatment of primary brown adipocytes produces a complete inhibition of insulin-stimulated glucose uptake and an impairment of the redistribution of GLUT4 from internal membranes to plasma membranes in response to insulin.

Earlier studies with staurosporine suggested that it completely inhibits both insulin- and phorbol 12-myristate 13-acetate–stimulated glucose transport activities in rat adipose cells (31, 47). In the present study, we have demonstrated that UCN-01 mainly exerts its suppression of glucose transport capacity by reduction in Akt activity and GLUT4 translocation. Most importantly, UCN-01 reduces insulin-stimulated Akt activation; whether this inhibition is attributable to altered phosphatase activity, translocation to the membrane, or suppression of other signaling molecules such as IRS-1 remains to be established in future studies.

In conclusion, this work suggests that UCN-01 impairs in a concentration-dependent fashion glucose transport in adipose cells both at physiologic and at abnormally high insulin concentrations. Decreased Akt activity after exposure to UCN-01 can clearly explain many aspects of this effect and may be the primary reason why the cancer patients who are treated with UCN-01 develop insulin resistance. Additional research is required to clarify the mechanistic relationship between Akt, glucose transport, and insulin resistance, and UCN-01 may be a valuable tool in additionally understanding these relationships.

REFERENCES

7-Hydroxystaurosporine (UCN-01) Inhibition of Akt Thr<sup>308</sup> but not Ser<sup>473</sup> Phosphorylation: A Basis for Decreased Insulin-Stimulated Glucose Transport

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