Increased Protein Kinase C Activity Is Linked to Reduced Insulin Receptor Autophosphorylation in Liver of Starved Rats*

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Phosphorylation of the insulin receptor β-subunit on serine/threonine residues by protein kinase C reduces both receptor kinase activity and insulin action in cultured cells. Whether this mechanism regulates insulin action in intact animals was investigated in rats rendered insulin-resistant by 3 days of starvation. Insulin-stimulated autophosphorylation of the partially purified hepatic insulin receptor β-subunit was decreased by 45% in starved animals compared to fed controls. This autophosphorylation defect was entirely reversed by removal of pre-existing phosphate from the receptor with alkaline phosphatase, suggesting that increased basal phosphorylation on serine/threonine residues may cause the decreased receptor tyrosine kinase activity. Tryptic removal of a C-terminal region of the receptor β-subunit containing the Ser/Thr phosphorylation sites similarly normalized receptor autophosphorylation. To investigate which kinase(s) may be responsible for such increased Ser/Thr phosphorylation, protein kinase C and cAMP-dependent protein kinase A in liver were studied. A 2-fold increase in protein kinase C activity was found in both cytosol and membrane extracts from starved rats as compared to controls, while protein kinase A activity was diminished in the cytosol of starved rats. A parallel increase in protein kinase C was demonstrated by immunoblotting with a polyclonal antibody which recognizes several protein kinase C isoforms. These findings suggest that in starved, insulin-resistant animals, an increase in hepatic protein kinase C activity is associated with increased Ser/Thr phosphorylation which in turn decreases autophosphorylation and function of the insulin receptor kinase.

The insulin receptor possesses intrinsic tyrosine kinase activity which appears to be essential for signal transmission (1, 2). In patients with non insulin-dependent diabetes mellitus (NIDDM),1 receptor kinase activity is reduced by 40–80% (3–5). In obese patients with NIDDM, weight loss improves insulin resistance and the diabetic state and is associated with parallel improvement in the kinase activity (6), suggesting that the phosphorylation defect in these patients is caused by a regulatory rather than a genetic change in the receptor. We and others have shown that regulation of the intrinsic kinase activity at the receptor depends on the phosphorylation state of the β-subunit of the receptor (7, 8). Autophosphorylation of tyrosine residues increases kinase activity, whereas serine phosphorylation by other kinases leads to a decrease in the tyrosine kinase activity. Serine phosphorylation can be catalyzed by cAMP-dependent protein kinase (9) or by the Ca²⁺ and phospholipid-dependent protein kinase C (8–10).

Starvation induces insulin resistance (11) and, in parallel, a reduction in insulin receptor kinase activity (12–14). In this study, we have examined starvation-induced changes of the insulin receptor and protein kinase C systems in rat liver and found evidence in this model of insulin resistance that the decrease in receptor tyrosine kinase may be mediated through Ser/Thr phosphorylation of the receptor by protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—[32P]Orthophosphate, [γ-32P]ATP, and Triton X-100 were obtained from Du Pont-New England Nuclear; porcine insulin was from Elanco; and wheat germ agglutinin-agarose was from Vector Laboratories, Inc. The following chemicals were from Sigma: histone type IIa, histone V5, leupeptin, aprotinin, benzamidine, acetylglucosamine, alkaline phosphatase, Phosphatidylycerine and diolein was from Avanti Polar Lipids; CHAPSO was from Pierce Chemicals; Pipes, MOPS, and Pansorb were from Calbiochem; DEAE-cellulose (Sephacel) was from Pharmacia LKB Biotechnology Inc.; phosphocellulose papers (P81) were from Whatman; TPCF-treated trypsin was from Worthington; and all reagents for SDS-PAGE were from Bio-Rad. Immobilized alkaline phosphatase was prepared by reacting 2 ml of Aff-Gel 10 (Bio-Rad) with 5 mg (1000 units/mg) of alkaline phosphatase from bovine intestinal mucosa (Sigma, P-2276) as described in the manufacturer's instructions.

Preparation of Solubilized Insulin Receptor—Rats were killed by cervical dislocation, and livers were removed, washed with ice-cold saline, weighed, minced with scissors, and homogenized on ice in 3 volumes of 50 mM HEPES, pH 7.4, containing 100 mM NaF, 100 mM sodium pyrophosphate, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride in a Dounce homogenizer with a loose pestle. The homogenate

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1 The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus; CHAPSO, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazinediethanesulfonic acid; WGA, wheat germ agglutinin; EGTa, [ethylene bis(oxyethylene)tetraacetic acid; EGF, epidermal growth factor; TPCF, 1-toyamalido-2-phenylethyl chloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.

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was centrifuged at 1200 rpm in an RT6000B Sorval centrifuge to remove large clumps, and Triton X-100 was added to the supernatant to a final concentration of 1%. The resulting suspension was stirred for 60 min and centrifuged at 100,000 × g for 60 min, and the supernatant was applied to a 2-ml column of agarose-bound wheat germ agglutinin, all at 4°C. After extensive washing with 50 mM HEPS buffer, pH 7.4, 0.1% Triton X-100, the receptors were eluted with this buffer containing 0.05 M N-acetylglucosamine. Binding capacity of each preparation was determined as previously described (33), and all preparations were normalized for the same binding capacity.

**Phosphorylation with Solubilized Receptors**—Solubilized, lecithin-purified insulin receptors (2–4 μg of total protein) were diluted in a final volume maintaining the composition of PBS, pH 7.4, containing 0.1% Triton X-100, 4 mM MgCl₂. These mixtures were preincubated with or without insulin (10⁻⁷ M) at 22°C for 30 min. Phosphorylation was initiated by adding 200 μM [γ³²P]ATP and continued for 2 min at 22°C. The reaction was terminated by adding 1 ml of ice-cold 50 mM HEPS, pH 7.4, containing 0.1% Triton X-100, 100 mM sodium pyrophosphate, 5 mM EDTA, and 2 mM sodium vanadate. Phosphorylated insulin receptors were immunoprecipitated with affinity-purified anti-phosphotyrosine antibody preared as previously described (34). Anti-phosphotyrosine antibody (2 μg) was mixed with phosphorylated insulin receptor preparations and incubated for 2 h at 4°C. The antibody was adsorbed in Pansorbin (10%, 50 μl), and pellet was washed three times with 0.5 ml of 50 mM HEPES, pH 7.4, 1.0% Triton X-100, and 0.1% SDS. The phosphorylated proteins were eluted from the Pansorbin with Laemmli sample buffer containing 100 mM dithiothreitol at 100°C for 1 min, and the proteins were separated by SDS-PAGE using 7.5% resolving gels and were identified by autoradiography at -70°C with Cronex intensifying screens.

**Preparation of Liver Homogenates for Enzyme Assays**—All procedures of hormone assays were as described by Azhar et al. (20): 1 g of liver was homogenized in 30 ml of buffer containing 0.25 M sucrose, 20 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA, 0.2 mM PMSF, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM TLCK, 0.1 mM TPCK, 5 μg/ml aprotinin, and 0.5 mM benzamidine in a Potter-Evjehej homogenizer at 8000 rpm for 20 s at 4°C. The homogenate was centrifuged at 8000 × g for 20 min to pellet erythrocytes, intact nuclei, and mitochondria. The supernatant was separated into a microsomal pellet and cytosolic fraction by centrifugation at 200,000 × g for 45 min. The microsomal pellet was vigorously solubilized at 0°C in the same buffer containing 1% CHAPS and then centrifuged at 200,000 × g for 45 min. The supernatants containing cytosol and membrane extracts were added to 2 ml of DEAE-Sephacel (packed volume per g of liver) in Centrex centrifugal microfilter columns for 1 h at 4°C and after washing with a buffer also containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, and 10 mM dithiothreitol were eluted in the presence of 0.5 M NaCl.

**Assay of Calcium-activated and Phospholipid-dependent Protein Kinase**—Preparation of purified enzyme was carried out by a slight modification of the procedure of Corbin and Reimann (36). The assay system contained in a final volume of 100 μl: 50 mM MOPS, pH 7.0, 10 mM magnesium acetate, 1 mg/ml histone F1 (type V Sigma), 40 mM NaF, 0.25 mM [γ³²P]ATP (100–200 cpm/pmol), 1 mM theophylline, and a suitable aliquot of enzyme with or without 10 μM cAMP. The reaction was conducted for 10 min at 30°C and stopped as described above. The phosphocellulose strips were washed and processed for radioactivity determinations. Basal protein kinase activity was determined in the absence of cAMP. cAMP-dependent protein kinase was determined by subtracting the cAMP incorporation into histone noted in the absence of added cAMP from the amount of radioactivity incorporation noted in the presence of cAMP. Enzyme activity is expressed as pico moles of [³²P] transferred from [γ³²P]ATP histone per min per mg of protein.

**RESULTS**

**Autophosphorylation of the Insulin Receptor from Fed and Starved Rats**—To study starvation-induced changes in the activity of the insulin receptor tyrosine kinase, autophosphorylation of the receptor from livers of starved and ad libitum-fed rats was determined by affinity chromatography on WGA-agarose in the presence of phosphorylas/phosphothreonine phosphatase inhibitors: NaF (100 mM), Na₂PO₄ (10 mM), and EDTA (5 mM). Inulin binding capacity of the WGA-purified fractions was 130 ± 12 pmol and 190 ± 8 pmol of insulin/mg of protein for the fed (n = 4) and starved (n = 4) animals, respectively.

To compare receptor autophosphorylation, equal amounts of receptor (500 pmol of insulin binding capacity) from fed and starved animals were incubated with [γ³²P]ATP and Mn²⁺ in the absence and presence of 10⁻⁹ or 10⁻⁷ M insulin. In both preparations, the insulin-stimulated autophosphorylated receptor β-subunit was selectively immunoprecipitated with anti-phosphotyrosine antibody and appeared as a 95-kDa band on the autoradiograms of SDS gels (Fig. 1). Autophosphorylation of the insulin receptor from starved rats stimulated at both insulin concentrations was found to be decreased by an average of 48 ± 6% (n = 4) compared to that found in fed rats as determined by densitometry of the autoradiograms.

**Dephyosphorylation of Receptor by Alkaline Phosphatase or Mild Trypsinization Restores Its Activity**—To determine if the decrease in tyrosine autophosphorylation reaction might be related to an increase in the basal level of Ser/Thr phosphorylation of the WGA-purified receptor preparations, two approaches were used to remove pre-existing phosphate from

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the receptor prior to the autophosphorylation reaction. First, the WGA-purified receptors were incubated with immobilized alkaline phosphatase under conditions previously shown to remove from the receptor phosphate added by protein kinase C-catalyzed serine/threonine phosphorylation (8). WGA preparations treated with active or inactive alkaline phosphatase from fed and starved animals were subjected to insulin stimulation under the same conditions described above. De-phosphorylation of the insulin receptor caused an increase in insulin-stimulated autophosphorylation of the P-subunit of the insulin receptor from livers of both fed and starved rats. The increase was almost 4-fold (355%) in receptors from starved animals and only 2-fold (182%) in receptors from fed animals. Thus, the treatment with alkaline phosphatase reversed the defect in phosphorylation found on receptors of starved animals, such that insulin-stimulated autophosphorylation between fed and starved was 26 ± 6% before phosphorylation was detected in the absence of insulin. These autoradiographs were scanned, and the bar graph summarizes the results of four such experiments (mean ± S.D.).

Second, the WGA preparations were incubated with trypsin under conditions previously shown to remove a 10-kDa fragment from the C terminus of the β-subunit of the insulin receptor (15). This 10-kDa peptide contains threonine-1336 (16) which has recently been identified as a phosphorylation site by protein kinase C, and also contains at least one of the serine sites of phosphorylation (18). The trypsin-treated WGA-purified receptor preparation from liver of fed and starved rats were stimulated with 100 nM insulin under conditions identical with those described above and compared to each other as well as to non-trypsin-treated preparations. Trypsin-treated β-subunit migrated at Mₐ = 85, about 10,000 lower than the nondigested β-subunit. As in the dephosphorylation with alkaline phosphatase, removal of the Mₐ = 10,000 peptide normalized the decrease in autophosphorylation of the β-subunit seen in starved animals equalizing it to that of fed controls (Fig. 3).

Determination of Serine/Threonine Kinase Activity in Liver

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FIG. 1. Autophosphorylation of the insulin receptor purified from starved and fed rats. Equal amounts (500 pmol of insulin binding capacity of WGA-purified insulin receptor from liver of rats starved for 72 h and fed ad libitum, were incubated with 10⁻⁸ or 10⁻⁹ insulin. The phosphorylation reaction was started by adding [γ-³²P]ATP and Mn⁺⁺ and terminated after 2 min. The insulin receptor was immunoprecipitated with anti-phosphotyrosine antibody, analyzed on SDS-PAGE gels, and visualized by autoradiography. The autoradiographs are shown in the upper part, and results of scanning of four pairs of animals are summarized in the graph (in relative units).

FIG. 2. Autophosphorylation of the insulin receptor purified from starved and fed rats before and after treatment with alkaline phosphatase. Autophosphorylation of the WGA-purified insulin receptor from livers of starved and fed rats before and after incubation of the receptor with alkaline phosphatase-agarose for 1 h at 4 °C to remove all phosphate added by protein kinase C activation. The receptors were phosphorylated as described in the legend to Fig. 1. The two first groups of bars on the left are experiments performed with the intact receptor; the two groups of bars on the right are with receptor that was dephosphorylated by alkaline phosphatase. In each case, the solid bars represent the stimulation with 1 nM insulin; the hatched bar the stimulation with 100 nM insulin. In all cases, no phosphorylation was detected in the absence of insulin. These autoradiographs were scanned, and the bar graph summarises the results of four of such experiments (mean ± S.D.).

FIG. 3. Autophosphorylation of the insulin receptor purified from starved and fed rats before and after mild trypsinization. Autophosphorylation of the WGA-purified insulin receptor from liver of fed and starved rats was performed as in Fig. 1 (first two lanes). This experiment was repeated after incubation of the receptor with TPK-treated trypsin for 1 min at 22 °C and stopping the trypsinization with aprotonin before autophosphorylation (last two lanes). All lanes are of receptors stimulated with insulin. The experiment was repeated twice in two pairs of animals. The difference in autophosphorylation between fed and starved was 26 ± 6% before trypsinization and −5 ± 4% after removal of the 10-kDa piece from Fed and Starved Rats—The results of the autophosphorylation experiments shown above suggest that increased serine/threonine phosphorylation of the β-subunit may be the cause of the decrease in autophosphorylation of the insulin receptor. Two serine kinases have been implicated in phosphorylation of the insulin receptor on serine/threonine residues: cAMP-dependent protein kinase (proteins kinase A), and calcium- and phospholipid-dependent protein kinase (protein kinase C). Therefore, we measured the activity of these two enzymes following starvation. Livers of both starved and fed rats were homogenized in the presence of multiple protease inhibitors and the kinases were partially purified on a DEAE-Sepharose (see "Experimental Procedures").

In both fed and starved animals, protein kinase C activity in liver was higher in membrane as compared to cytosol (Fig. 4). Protein kinase C activity was higher in solubilized liver membranes of starved rats compared to fed controls (81 ± 17

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against a peptide corresponding to sequence 280-292 of brain protein.

Membrane was solubilized using CHAPS in order to unmask protein kinase C activity. Proteins were further purified on a DEAE-Sephacel column. Assay of protein kinase A and protein kinase C were performed as described under "Experimental Procedures." The bar graph summarizes results in 12 pairs of animals, and results are expressed in picomoles of phosphate/mg of protein/min.

Unlike protein kinase C, protein kinase A was decreased by starvation in rat liver. Cytosolic protein kinase A activity decreased from 42 ± 10 pmol of 32P incorporated/mg of protein/min to 21 ± 2.4 pmol of 32P/mg of liver during starvation rats.

**Immunoblotting of Protein Kinase C**—To determine whether the increased protein kinase C activity in liver of starved rats was due to increased amounts of the enzyme or increased specific activity, we measured the amount of immunodetectable protein kinase C in liver extracts from both fed and starved rats. Cytosolic and membrane extracts from four fed and starved rats were pooled, and samples containing equal amounts of protein were analyzed by SDS-PAGE and electroblotted to nitrocellulose. Probing was done using polyclonal antibody against peptide 0442 of bovine brain protein kinase C (19), and visualization was with 125I-protein A and autoradiography. In contrast to previous reports which suggested that protein kinase C is of lower M, in liver (20), we identified an undegraded form with M, = 82, equal to that found in brain (Fig. 5). In addition, liver contained multiple lower M, bands which are presumably proteolytic degradation products. As shown in Fig. 5, there was a 180% increase (by densitometry) in the nondegraded form of protein kinase C membrane extracts of starved animals. However, the degraded form of M, = 55,000 was more abundant in fed rat liver membrane extracts. Similar results were seen in cytosol where protein kinase C migrated at the same M, as in the membrane fractions and the M, = 82,000 band was more abundant (200%) in starved animals compared to fed animals.

**DISCUSSION**

Regulation of transmembrane signalling by receptor phosphorylation is a mechanism common to several different receptor systems including the family of receptors which are tyrosine kinases. The tyrosine kinase activity of both the EGF and insulin receptors are under positive and negative control by the state of receptor phosphorylation. Autophosphorylation of the insulin receptor on tyrosine residues enhances tyrosine kinase activity (7). In contrast, activation of both protein kinase A and protein kinase C in intact cells leads to Ser/Thr phosphorylation (4). Phosphorylation causes activation by protein kinase C, and this phosphorylation leads to a 65% decrease in its protein tyrosine kinase activity (10). A similar reduction is seen in insulin receptor purified from hepatoma cells which have been pretreated with a protein kinase C activator, and, in this case, treatment of the receptor tyrosine kinase with alkaline phosphatase reverses protein kinase C-mediated receptor phosphorylation and increases receptor kinase activity (8). In the case of the EGF receptor, the regulatory role of tyrosine phosphorylation remains debated, but serine/threonine phosphorylation, catalyzed at least in part by protein kinase C, reduces kinase activity and alters receptor binding (23). Threonine-654, located in the EGF receptor cytoplasmic domain, appears to be a major site of protein kinase C phosphorylation.

In this study, the role of serine/threonine phosphorylation in the starvation-induced decrease in the tyrosine kinase activity of rat liver insulin receptor was examined. In agreement with other reports (12-14), we found that insulin-stimulated autophosphorylation of insulin receptor from liver of rats starved for 72 h was decreased by ~50% compared to that of animals fed ad libitum. To probe the possibility that the reduced kinase activity seen in starved animals was a result of phosphorylation on serine/threonine residues, the in vitro autophosphorylation was repeated with receptors where pre-existing phosphate was removed, either by preincubation of the receptor with alkaline phosphatase or by removal of a 10-kDa piece of the C terminus of the β-subunit containing threonine-1336. The latter experiment was based on the recent work by Lewis et al.2 suggesting that protein kinase C phosphorylates the insulin receptor at threonine-1336 adjacent to the C terminus of the cytoplasmic portion of the β-subunit of the receptor, as well as on previous reports that found an increased phosphorylation on serine residues after phosphorylation of the insulin receptor by the purified kinase C in a cell free system (10) or after treatment of cells by phorbol esters (8). Both enzymatic manipulations led to a normalization or equalization of insulin-stimulated autophosphorylation of the receptor, suggesting that phosphate on Ser/Thr residues of the receptor could explain the decrease...
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in tyrosine kinase level and that the model of regulation by multisite phosphorylation may be valid in the intact animal. Starvation-induced insulin resistance is not the only reversible state of insulin resistance accompanied by a reversible decrease in insulin receptor kinase activity. In obese subjects with NIDDM, there is a 50-80% reduction in kinase activity of the insulin receptor from adipocytes, and this can almost be completely reversed upon weight loss. This correlates with correction of the glucose disposal rate as measured during a euglycemic clamp (6). This reversible decrease was suggested to result from an increased proportion of receptors that bind insulin but lack tyrosine kinase activity, rather than a diminished kinase activity of all the receptors (21). Although not tested, the increase in proportion of tyrosine kinase-deficient receptors in that study could well be a result of increased serine phosphorylation by protein kinase C. Increased protein kinase C activity alone or combined with a genetic propensity to an increase in Ser/Thr phosphorylation of the receptor, could lead to the altered receptor function observed in patients with NIDDM.

The defect in kinase activity of receptor purified from livers of streptozotocin diabetic rats is another example of a reversible state of insulin resistance, which in this case is partially restored by treatment of the rats with insulin (22). In data not shown, we performed a similar set of experiments to those used to characterize the starved animals and were unable to document similar changes. While we found a reduction in the receptor autoposphorylation, this defect was not reversed by alkaline phosphatase treatment, nor was protein kinase C activity in liver increased. Thus, the proposed model of regulation of the insulin receptor kinase may not be applicable to all forms of acquired or genetic models of insulin resistance in animals.

The finding of an increase in activity of protein kinase C in liver extracts of starved rats was not due to an increase in the activity of a nonspecific serine phosphatase, as starvation induced an opposite effect on protein kinase A. The higher protein kinase C activity was paralleled by an increase in the amount of the intact enzyme as demonstrated by immunoblotting. An opposite effect was seen in the amount of the major degradation products of protein kinase C as well as a change in the pattern of degradation. These results could be explained by a difference in proteolytic activity toward protein kinase C in livers of starved rats, or alternatively, they can be explained by a differential induction of protein kinase C isoenzymes by starvation. Protein kinase C activity is present in at least seven different isozymes (24–28). Each of these has a molecular weight ~80,000 and is composed of a M, = 50,000 catalytic domain and a M, = 30,000 regulatory domain. Recently, it has been shown that the cleavage of these different isozymes by trypsin and calpain differs with Type III being the most resistant (25, 26). The proteolytic fragments generated include a 60-kDa fragment which is fully active, but is no longer regulated by diacylglycerol, Ca ++ , and phospholipids, as well as 67- to 74-kDa fragments which retain partial dependence on these co-factors (29). Although the antibody we used recognizes at least three isoenzymes, if starvation induces production of one isoenzyme which is differentially proteolyzed, this could account for the findings regarding protein kinase C in liver.

Rapid and extensive degradation of protein kinase C in liver extracts has led to several erroneous results. Previous work found only low activity in liver compared to other organs on a weight basis (30). Azhar et al. (20) used mutiple protease inhibitors and a mild detergent to unmask a much higher activity of protein kinase C in rat liver. The same group purified protein kinase C and determined it to be composed of subunit with molecular weight of 64,000 and described three isoenzymes with nearly identical enzymatic properties. In this work, we were able to demonstrate by immunoblotting that the intact protein kinase C in liver is of identical molecular weight as that in brain and that the previously described 64-kDa subunits of the enzyme are probably degradation products.

Protein kinase C is thought to play a major role in control of a wide variety of processes (28). Increased activity in starvation would certainly have a broader influence beyond the effects described here. For example, the EGF receptor in hepatocytes of starved rats is reported to have decreased binding and tyrosine kinase activity (31) that could result from starvation-induced protein kinase C activation. Phosphorylation of glycogen synthase by protein kinase C has been shown to lead to its inactivation (32) and may be involved in the decrease in glycogen synthase activity in starvation.

Although several experimental lines of evidence reported in this study support the hypothesis that the model of regulation of insulin receptor tyrosine kinase in vitro is correct for the intact animal, direct evidence is still lacking. This task awaits direct measurements of phosphoserine and phosphothreonine levels in hepatic insulin receptors.

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