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Guanine Nucleotide Binding Regulatory Proteins in Liver From Obese Humans With and Without Type II Diabetes: Evidence for Altered "Cross-Talk" Between the Insulin Receptor and G_i-Proteins

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Abstract A novel pathway for physiological "cross-talk" between the insulin receptor and the regulatory G_i-protein has been demonstrated. We tested the hypothesis that a coupling defect between G_i and the insulin receptor is present in the liver of obese patients with and without type II diabetes. Insulin 1×10^{-9} M ($\sim ED_{50}$) and 1×10^{-7} M (Max) inhibited pertussis toxin-catalyzed ADP ribosylation of G_i in human liver plasma membranes from lean and obese nondiabetic patients. However, 1×10^{-7} M insulin was without effect in membranes from patients with type II diabetes. This coupling defect was not intrinsic to G_i, since Mg^{2+} and GTPγS inhibited pertussis toxin-catalyzed ADP ribosylation in both diabetic and nondiabetic patients. Binding of insulin of the α-subunit and activation of the tyrosine kinase intrinsic to the β-subunit of the insulin receptor are not responsible for the coupling defect. ^{125}I insulin binding is the same in obese patients with or without diabetes. Tyrosine kinase of the insulin receptor is decreased in diabetes. However, a monoclonal antibody to the insulin receptor (MA-20) at equimolar concentrations with insulin equally inhibits pertussis toxin-catalyzed ADP ribosylation of G_i without activating tyrosine kinase or insulin receptor autophosphorylation. Immunodetection of G-proteins suggested that $G_{i3\alpha}$ was normal in diabetes and $G_{i1\text{-}2\alpha}$ was decreased by 40% in the diabetic group as compared to the obese nondiabetic group but was normal when compared to the lean non diabetic group. We conclude that the novel pathway of insulin signaling involving the regulatory G_i proteins via biochemical mechanisms not directly involving the tyrosine kinase of the insulin receptor is altered in obese type II diabetes and offers a new target for the search of the mechanism(s) of insulin resistance.

Key words: diabetes, G proteins, insulin, obesity, insulin receptors

INTRODUCTION

Many hormones and neurotransmitters use specific receptors that interact noncovalently with guanine nucleotide binding proteins (G-proteins) in the transmembrane signaling process [Casey and Gilman, 1988; Neer and Clapham, 1988; Spiegel, 1987; Stryer and Bourne, 1986]. Despite many attempts to understand the mechanism of insulin action [De Fronzo, 1988; Goldfine, 1987; Kaha and White, 1988; Larner, 1988; Olefsky et al., 1988; Reaven, 1988], the coupling of the insulin receptor with distinct G-proteins remains to be established. The recent demonstration that insulin inhibits pertussis toxin (PTX)-catalyzed ADP-ribosylation of G_i in rat liver plasma membranes [Rothenberg and Kahn, 1988], however, suggests a functional "cross-talk" between the insulin receptor and the inhibitory guanine nucleotide-binding protein (G_i). In addition, a dramatic reduction of over 90% in G_i has been reported to occur in liver plasma membranes obtained from rats made diabetic by either alloxan or streptozotocin [Gawler et al., 1987]. This reduction, however, has not been confirmed in the liver of

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rats with streptozotocin-induced diabetes [Lynch et al., 1989]. Only a modest decrease of PTX-catalyzed ADP ribosylation of G_i has been demonstrated in acute spontaneously diabetic (BB/WOR) rats [Lynch et al., 1989].

G_i proteins comprise three distinct molecular species [Itoh, 1988], which are involved not only in regulation of adenylate cyclase [Katada et al., 1987] but also in activation of phospholipases [Ohta et al., 1985], gating of potassium [Pfaffinger *et al.*, 1985], calcium channels [Holz et al., 1989], and cells proliferation [Murayama and Ui, 1987]. Thus, these proteins are particularly significant in diabetes because some of the metabolic pathways in which G_i is involved may be altered in this disease [Caro et al., 1989; Houslay et al., 1989; Thakkar et al., 1989].

The purpose of this study is to investigate possible alterations in the immunodetection of G-proteins and in the functional "cross-talk" between the insulin receptor and G_i-proteins in liver plasma membranes from humans with and *without* type II dabetes. The hypothesis that a coupling defect between G_i and the insulin receptor present in the livers of obese patients with and without type II diabetes is tested.

METHODS

Patients

The study group consisted of 8 morbidly obese patients with type II diabetes, 8 morbidly obese patients without diabetes admitted to the hospital to undergo gastric bypass as treatment for morbid obesity, and 8 nonobese, nondiabetic patients admitted for elective cholecystectomy. Except for obesity and diabetes, none of the patients had other diseases or were taking medications known to alter carbohydrate metabolism. Two diabetic patients were receiving sulfonylureas and two other diabetic patients were taking insulin, which were discontinued 2 weeks and 1 week before the day of surgery, respectively. All patients had maintained constant body weight during the months preceding hospital admission and received a weight-maintaining diet providing 50% of total calories as carbohydrates, 30% as fat (polyunsaturated/saturated fat ratio of 0.4, cholesterol content of 100 mg), and 20% as protein for 1 week before surgery. Each morbidly obese patient without known diabetes mellitus had a 75-g oral glucose tolerance test, as recommended by the National Diabetes Data Group, in order to classify them as nondiabetics. Written consent was obtained from each patient after they were informed about the nature, potential benefit, and potential risk associated with the study. The patients underwent surgery after an overnight fast and only intravenous saline was given before the liver biopsy. After opening and exploring the abdomen, a 1–2-g liver biopsy specimen was obtained from the left lobe of the liver in order to prepare plasma membranes, cytosol, and solubilized insulin receptors.

Preparation of Liver Plasma Membranes

Plasma membranes from human liver were prepared, with minor modifications [Caro et al., 1987b], by the aqueous polymer method of Lesko et al. [Lesko et al., 1973]. Liver tissue was minced into small pieces and suspended in homogenization buffer A consisting of 0.5 mM $CaCl_2$, 1 mM $NaHCO_3$, pH 7.0, 1 mM sodium orthovanadate, 4 mM EDTA, 1 mM phenylmethylsufonyl fluoride (PMSF), 1,000 U/ml aprotinin, 2 μM leupeptin, 2 μM pepstatin, and 0.1 mg/ml bacitracin. The tissue was gently homogenized with 25 strokes in a Dounce homogenizer, diluted 100-fold with buffer A, and centrifuged at 2,600*g* for 30 min. The supernatant was discarded and the pellet washed several times with decreasing volumes of buffer A. The pellet was then gently mixed with 10 ml of dextran/polyethylene glycol polymers and centrifuged at 1,100*g* for 30 min. The membrane fraction at the polymer interphase was collected and washed three times with buffer A. The membranes were then suspended in 50 mM Hepes, pH 7.5, which contained the protease inhibitors listed in buffer A at 10 mg/ml protein concentration in the same buffer, snap frozen, and stored at −70°C until used. The membranes used in these studies were 10-fold enriched in terms of Na^+,K^+-ATPase [Arner et al., 1987], as compared to the homogenate, and contained functional insulin receptors capable of promoting phosphorylation of its β-subunit and other endogenous membrane substrates, as demonstrated previously [Caro et al., 1987b].

Preparation of Liver Cytosol

After preparation, liver homogenate was centrifuged at 7,000*g* for 30 min. The resulting supernatant was centrifuged at 105,000*g* for 120 min. The pellet was discarded and the supernatant was aliquoted, snap frozen, and stored at −70°C until used.

Preparation of Solubilized Insulin Receptor

Partial purification of the insulin receptor from human liver was performed according to the procedure described previously [Caro et al., 1986]. Frozen liver was powdered under liquid nitrogen, weighed, and homogenized as a frozen slurry in 50 mM Hepes, pH 7.4, containing 1% Triton X-100, 2 μM pepstatin, 2 μM leupeptin, 1 mM PMSF, 0.1 mg/ml bacitracin, 1,000 U/ml aprotinin, and 1 mM sodium orthovanadate. The homogenate was solubilized by stirring for 60 min at 4°C. The mixture was then centrifuged at 100,000*g* for 60 min, and the crude solubilized extract was partially purified by wheat germ agglutinin affinity chromatography. Recovery of ^{125}I-insulin binding activity was over 85% in a partially purified preparation [Caro et al., 1986].

ADP-Ribosylation of G-Proteins by Pertussis Toxin and Cholera Toxin

ADP-ribosylation of liver plasma membranes and cytosol fraction was performed as described by Ribeiro-Neto et al. [1985], with the minor modifications described by Rothenberg and Kahn [1988]. In a typical assay, 20 μl of liver plasma membrane, corresponding to 50 μg protein, was incubated at 30°C in a 100-μl final volume containing 10 mM arginine, 10 mM thymidine, 10 mM NADP, 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, 0.5 mM EGTA, 1 mM $MgCl_2$, 0.5 mM DTT, 10 mM Tris-HCl, 0.01 mM (a-^{32}P) NAD (2–5 μCi), and 1 μg pre-activated PTX or 1 μg CTX (subunit A). Incubation times varied and the reaction terminated by the addition of 1 ml 20% (w/v) trichloroacetic acid. After 20 min at 4°C, the precipitates were pelleted in a microfuge (10 min at 14,000*g*). The supernatants were discarded and the pellets washed twice with 1 ml of ethyl ether. The pellet was then dissolved in Laemmli buffer [Laemmli, 1970], heated in boiling water bath for 3 min, and then loaded onto a 10% polyacrylamide gel with 3.75% polyacrylamide stacking gel. After electrophoresis, the gels were fixed for 4 h in 50% methanol and 7.5% acetic acid, followed by staining in 0.2% (w/v) Coomassie Brilliant Blue R, 50% (v/v) methanol, and 10% (v/v) acetic acid for 10 min. The gels were destained in 15% (v/v) methanol and 7.5% (v/v) acetic acid until the background was clear. The gels were dried and examined by autoradiography for 70 h at −70°C, using Kodak X-Omatic film in a Kodak X-Omatic cassette with intensifying screens. The autoradiographic plates were then scanned with an LKB ultroscan densitometer with Gelscan XL software.

Immunodetection of G-Proteins

Proteins from human liver plasma membranes (50 μg) were resolved by 10% polyacrylamide gel electrophoresis–sodium dodecyl sulfate (PAGE–SDS) system on 5 cm × 5 cm slab gels for 2 h at 100 V. Proteins were transferred to immobilon P nylon membranes using a Millipore Semidry blotting apparatus. The membranes were blocked for 2 h with 5% Blotto and then incubated overnight at 4°C with polyclonal antibodies raised in rabbits against synthetic C-terminal decapeptides of the α-subunit of $G_{i1-2\alpha}$, $G_{i-3\alpha}$, and G_s [Lynch et al., 1989]. The antibodies were partially purified by affinity chromatography using immobilized protein A columns, and were used at a concentration of 2 μg/ml in 5% Blotto. The blots were then washed with TBS buffer with and without Tween-20 and were probed with ^{125}I-goat antirabbit IgG overnight. Autoradiography was carried out for 48 h at −70°C, and the resulting autoradiograph was analyzed by densitomery. Detection of G-proteins was linear over a protein concentration range of 10–100 μg.

Immunoprecipitation of G-Proteins

Following ADP-ribosylation in both the presence and absence of insulin (1×10^{-7} M), human liver plasma membranes were solubilized for 1 h at 4°C in 50 mM Tris–Cl pH 7.5 with 1% Triton X-100 containing the protease inhibitors used for solubilization of insulin receptors. The solubilized proteins were diluted (1:4) with 50 mM Tris–Cl pH 7.5 without Triton X-100 and centrifuged at 100,000*g* for 60 min. Polyclonal antibodies to $G_{i1-2\alpha}$ and $G_{i3\alpha}$ 2 μg/ml concentrations were added to the supernatants and incubated for 16 h at 4°C. A 10% (w/v) suspension of Pansorbin was added to the immunoprecipitation reaction mixture and the incubation was continued for an additional 1 h at 4°C. After completion, the Pansorbin was sedimented by centrifugation at 500*g*. The pellet was washed once with 50 mM Hepes buffer, pH 7.4, containing 0.1% Triton X-100, washed again with 50 mM Hepes buffer pH 7.4, containing 0.5 M NaCl, and finally washed with 50 mM Hepes buffer, pH 7.4, containing 0.01% SDS. The pellet was then suspended in Laemmli buffer [Laemmli, 1970] and boiled for 5 min. The

Pansorbin was sedimented by centrifugation and the supernatant subjected to SDS–PAGE.

Insulin Receptor Tyrosine Kinase Activity

Partially purified insulin receptors (20 μg protein) were incubated both in the presence and absence of 1×10^{-7} M insulin at 4°C for 16 h. Following incubation, tyrosine-specific protein kinase activity was determined at room temperature by the addition of [^{32}P]ATP (2 μCi, 100 μM) in the presence of 2.5 mg/ml, Glu^4-Tyr^1, 10 mM $MgCl_2$, and 0.5 mM $MnCl_2$ in 50 mM Hepes buffer, pH 7.4 as previously reported [6]. The reaction was stopped 30 min later with 10% TCA containing 10 mM pyrophosphate and 3 mg/ml bovine serum albumin (BSA) and processed as previously described [Caro et al., 1986].

Autophosphorylation of the insulin receptors was initiated by adding [^{32}P]ATP (20 μCi, 5 μM) in the presence of 5 mM $MgCl_2$ and 10 mM $MnCl_2$ in 50 mM Hepes buffer, pH 7.4, and continued for 60 min at 4°C for at least 120 min because the reaction was linear. The reaction was then stopped with 50 mM Hepes buffer containing 10 mM EDTA, 100 mM NaF, 20 mM pyrophosphate, and 4 mM ATP. This mixture was incubated with 1/200 dilutions of polyclonal insulin receptor antibodies raised in rabbits against rat liver insulin receptors. After incubation at 4°C for 6–10 hs, the immunoprecipitates were separated using Pansorbin as previously described [Caro et al., 1986].

RESULTS

Table I lists the clinical characteristics of the three groups of patients who were all comparable as to age and gender. Patients with type II diabetes and morbid obesity were compared to morbidly obese patients declared to be nondiabetic by a normal oral glucose tolerance test. A nonobese group of patients without diabetes was also studied in order to investigate any effect that obesity itself might have on the results of the experiments.

TABLE I. Clinical and Biochemical Data of Study Subjects*

	Lean control	Obese control	Type II obese diabetes
Age (yr)	34 ± 3	36 ± 4	40 ± 6
Gender	5F.3M	6F.2M	6F.2M
Body mass index (kg/m^2)	23 ± 3	41 ± 6	40 ± 4
Plasma glucose (mg/dl)	78 ± 8	88 ± 4	202 ± 24
Plasma insulin (μU/ml)	11 ± 4	29 ± 8	35 ± 10

*Numbers are mean ±SEM.

Initial experiments were performed to characterize the methods in human liver. Incubation of 100 μg of liver cytosol or plasma membranes with PTX or CTX in the presence of [α-^{32}P]NAD resulted in ADP ribosylation of different proteins. In plasma membranes 41-kD and 45-kD proteins were predominantly ADP-ribosylated by PTX and CTX, respectively, which have been demonstrated in other cell systems to correspond to α-subunits of G_i and G_s [Casey and Gilman, 1988; Neer and Clapham, 1988; Spiegel, 1987; Stryer and Bourne, 1986], respectively. In the cytosol fraction, these proteins were not ADP-ribosylated but other low molecular weight proteins were found to be substrates for PTX-catalyzed ADP-ribosylation, whereas only a 25-kD protein was a substrate for CTX-catalyzed ADP-ribosylation. These low-molecular-weight proteins were not investigated further and may belong to a newly discovered family of small G proteins [Burgoyne, 1989].

Because Rothenberg and Kahn [1988] demonstrated that insulin inhibits PTX-catalyzed ADP-ribosylation of G_i in rat liver plasma membranes, we examined the behavior of G_i in human liver. PTX-catalyzed ADP-ribosylation was dependent on time and protein concentration. PTX-catalyzed ADP-ribosylation was also significantly reduced by the addition of Mg^{2+} in a dose-dependent manner. ADP ribosylation was inhibited >50% by 10 mM Mg^{2+} and >90% by 50 mM Mg^{2+}. GTPγS, a nonhydrolyzable analog of GTP, exhibited a similar effect on PTX-catalyzed ADP-ribosylation of G_i Mg^{2+} and GTPγS have both been shown to promote dissociation of G_i to a form that cannot support labeling by PTX [Casey and Gilman, 1988; Neer and Clapham, 1988; Spiegel, 1987; Stryer and Bourne, 1986].

These experiments (data not shown) establish that some of the known regulators of PTX-catalyzed ADP-ribosylation of G_i are operative in human liver plasma membranes. We and others have demonstrated the structure and functional integrity of the insulin receptor in these plasma membranes, and the integrity of the coupling between phosphatidylinositol-4,5-bisphosphate specific phospholipase C and adenylate cyclase with hormone and metabolic regulators [Caro et al., 1987b; Livingston et al., 1985; Thakkar et al., 1989]. If a functional "cross-talk" exists between insulin receptors and G_i as previously demonstrated [Rothenberg

and Kahn, 1988], our experimental system should be appropriate to study this phenomenon in liver from patients with and without type II diabetes. Figure 1 demonstrates that the addition of insulin 1×10^{-7} M to the reaction mixture results in a rapid decrease of PTX-catalyzed ADP-ribosylation of G_i. When the reaction was conducted at 37°C, it was noted that the inhibition of insulin was maximum at the earliest time studied (2.5 min), followed by progressive disappearance of its effect. When the same experiments were conducted at 30°C, the insulin effect was linear and could be observed up to 1 h after incubation (Fig. 2). Subsequent experiments, then, were performed at 30°C for 20 min. Figure 3 demonstrates that the insulin effect is dose dependent, with 50% inhibition at approximately 1×10^{-9} M insulin and maximal inhibition at 1×10^{-7} M insulin. This is consistent with the insulin dose–response curve of other biological functions in the human liver when these are studied in vitro [Caro et al., 1986].

An important consideration in these studies is that PTX-catalyzed ADP-ribosylation occurs in three distinct molecular species of G_i-protein α-subunit [Itoh et al., 1988]. Other G-proteins

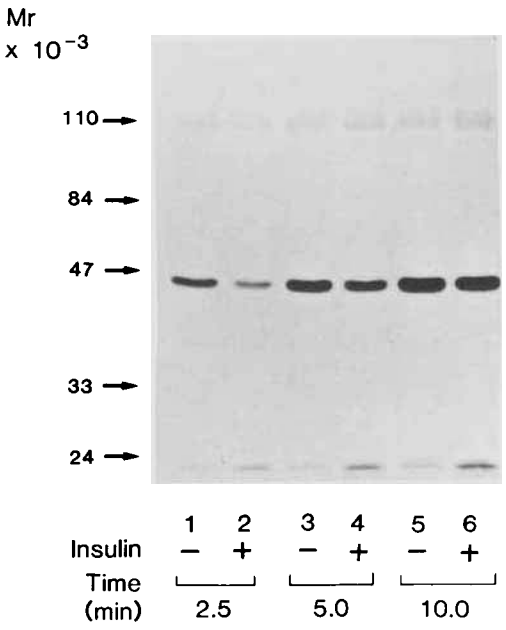

Fig. 1. Effects of insulin on pertussis toxin catalyzed ADP-ribosylation of G_i in human liver plasma membranes at 37°C. Liver plasma membrane (50 μg) was incubated with or without insulin (1×10^{-7} M) in the presence of pertussis toxin at 37°C for the times indicated and ADP-ribosylation, SDS-PAGE, and autoradiography were performed as described in Methods. The experiments are representative of three experiments from different liver membrane preparations.

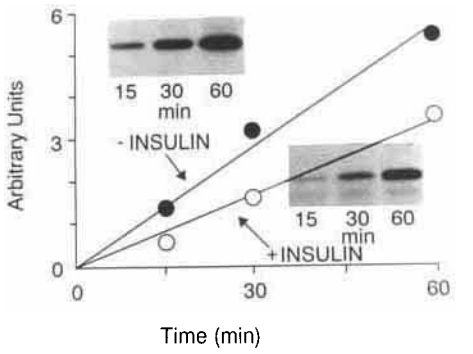

Fig. 2. Effect of insulin on pertussis toxin catalyzed ADP-ribosylation of G_i in human liver plasma membrane at 30°C. The experiment was performed as described in legend of Figure 1 but at 30°C. The experiment was reproduced one time using different liver plasma membranes.

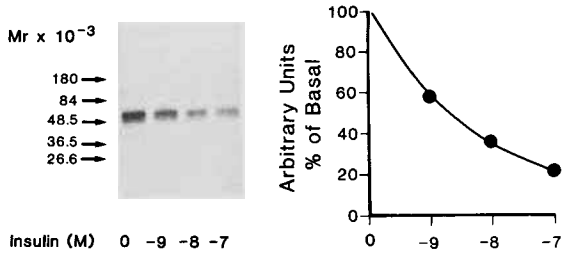

Fig. 3. Insulin dose response for inhibition of pertussis toxin catalyzed $G_i\alpha$ ADP-ribosylation of G_i. Pertussis toxin catalyzed ADP-ribosylation of $G_i\alpha$ was studied in human liver plasma membrane (50 μg) in the presence and absence of different insulin concentrations at 30°C for 20 min. **Left:** Representative autoradiograph. **Right:** Quantification of the data by gel densitometer from three separate experiments (mean ± SEM).

that are substrates for PTX, such as $G_{o\alpha}$, $G_{\tau\alpha 1}$, and $G_{\tau\alpha 2}$, are not present in liver [Casey and Gilman, 1988; Neer and Clapham, 1988; Spiegel, 1987; Stryer and Bourne, 1986]. It is possible then, that the insulin inhibition of PTX-catalyzed ADP-ribosylation is specific to one or more of the G_i-proteins and that this subset of G_i-protein might be decreased in diabetes. To address this possibility, the plasma membranes were solubilized with Triton-X 100, followed by immunoprecipitation with specific antibodies against $G_{i1\text{-}2\alpha}$, and $G_{i3\alpha}$ as described under Methods. Figure 4 shows that antibodies against $G_{i3\alpha}$ immunoprecipitated a 41-kD protein that is ADP-ribosylated by PTX and that this reaction is inhibited by insulin. Antibody $G_{i1\text{-}2\alpha}$ also immunoprecipitated a protein of identical molecular weight in which ADP-ribosylation is also inhibited by insulin. When both antibodies were used together, the radioactivity associated with the immunoprecipitated protein is similar to the sum recorded when the antibodies were used separately. The total amount of ADP-ribosylated

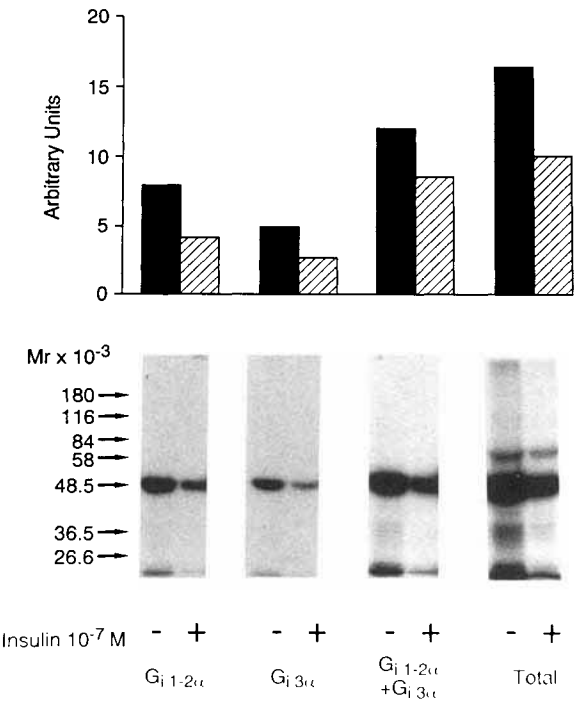

Fig. 4. Effect of insulin on pertussis toxin catalyzed ADP-ribosylation of $G_{i1\text{-}2\alpha}$ and $G_{i3\alpha}$. ADP-ribosylation in the presence and absence of insulin (1×10^{-7} M) was performed as described in legend of Figure 1. Proteins were then solubilized and precipitated either with $G_{i1\text{-}2\alpha}$ or $G_{i3\alpha}$ antibodies or with 10% trichloroacetic acid. The experiment was reproduced one time using different plasma membranes.

proteins precipitated by 10% trichloracetic acid, however, was about 20% greater, which demonstrates either the efficiency of the antibodies or that other proteins besides $G_{i1\text{-}2\alpha}$ and $G_{i1\text{-}3\alpha}$ are ADP-ribosylated in human liver plasma membranes.

We studied the relative distribution of G_i-proteins in liver plasma membranes from patients with and without type II diabetes by Western blot analysis. It should be noted that, like ADP-ribosylation, immunodetection is not a quantitative method because the former is the result of a complex enzymatic reaction and the latter is dependent on the antigenic properties of the protein. The immunodetection of $G_{i3\alpha}$ in our patients is shown in Figure 5. The left panel of Figure 5 shows a representative autoradiography with immunodetection of liver $G_{i3\alpha}$ in a lean (L), obese (O), and obese diabetic patient (D). The right panel shows the quantification by gel densitometry of the experiments performed in six different patients in each group. Analysis of variance demonstrates no statistically significant differences in the data. It should be noted, however, there is modest obesity-related increase in $G_{i3\alpha}$. Figure 6 shows the immunodetection of liver $G_{i1\alpha}$ and $G_{i2\alpha}$ in the same lean, obese, and diabetic patients. Quantification of $G_{i1\text{-}2\alpha}$ by gel densitometry also shows an obesity-related

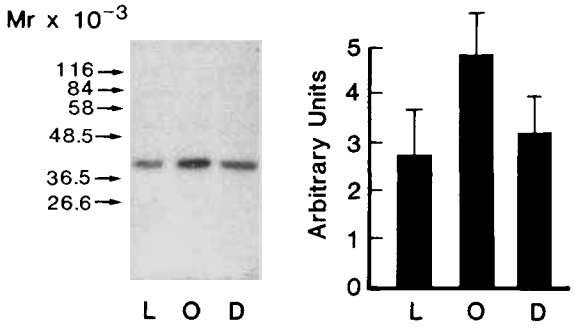

Fig. 5. Immunodetection of liver $G_{i3\alpha}$ in lean, obese, and diabetic patients. **Left:** Western blot of human liver plasma membranes (50 μg protein), as described in Methods, using antibodies that recognize $G_{i3\alpha}$ in a lean (L), obese (O), and obese diabetic patient (D). **Right:** Mean ± SEM of the data quantitated by gel densitometer from six different patients in each group.

increase in G_i. These differences were more pronounced for $G_{i1\text{-}2\alpha}$ and were statistically significant. $G_{i1\text{-}2\alpha}$ was higher in obese nondiabetic patients as compared to lean nondiabetic patients ($P < 0.01$) and obese diabetic patients ($P < 0.05$). No difference was noted between lean nondiabetic patients and obese diabetic patients.

An increase in G_s has been demonstrated in liver plasma membranes from chemically induced diabetes and BB/WOR rats [Lynch et al.,

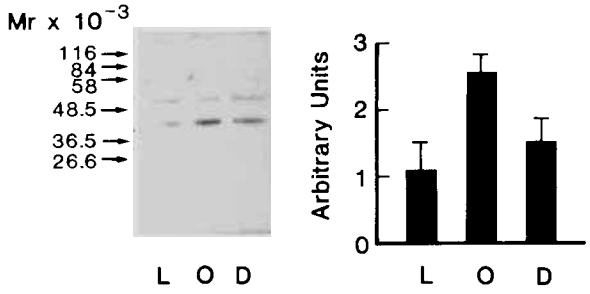

Fig. 6. Immunodetection of liver $G_{i1\text{-}2\alpha}$ in lean, obese, and diabetic patients. **Left:** Western blot of human liver plasma membranes (50 μg protein) was performed as described in Methods, using antibodies that recognize $G_{1\text{-}2\alpha}$ in a lean (L), obese (O), and obese diabetic patient (D). **Right:** Mean ± SEM quantitated by gel densitometer from six different patients in each group.

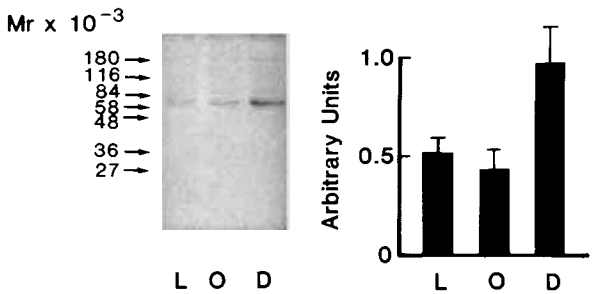

Fig. 7. Immunodetection of liver G_s in lean, obese, and diabetic patients. **Left:** Western blot of human liver *plasma membranes* (50 μg protein) as described in Methods, using antibodies that recognize G_s of a lean (L), obese (O), and obese diabetic patient (D). **Right:** Mean ± SEM of the data quantitated by gel densitometer from six different patients in each group.

1989]. G_s is also significantly increased ($P < 0.01$) in type II diabetic patients when compared to obese and lean nondiabetic patients (Fig. 7).

We believe that we have demonstrated a functional cross-talk between the insulin receptor and G_i-proteins in human liver, and only minor changes in the relative distribution of G_i immunodetection when diabetic and nondiabetic patients are compared. The next question to explore was whether the "cross-talk" between insulin receptors and G_i-proteins is altered by diabetes. The left panel of Figure 8 shows a representative autoradiograph of PTX-catalyzed ADP-ribosylation in the presence and absence of insulin in lean and obese nondiabetic patients and in obese patients with NIDDM. The right panel shows the quantification by gel densitometry of experiments performed with six different patients in each group. In the absence of insulin, PTX-catalyzed ADP-ribosylation of G_i was higher in the obese nondiabetic patients but this difference was not statistically significant. Insulin at 1×10^{-7} M concentrations, however, significantly inhibited PTX-catalyzed ADP-ribosylation in the lean nondiabetic patient ($P < 0.01$) and in the obese nondiabetic patient ($P < 0.05$) but showed no appreciable effect in obese patients with type II diabetes. Insulin effect was seen in all individual assays from non diabetic patients. This effect was variable, with the minimum of 9% inhibition noted in an obese patient. These data demonstrate, for the first time, a functional abnormality in the "cross-talk" between the insulin receptor and G_i-protein in diabetes.

Mg^{2+} and GTPγS are known to activate G_i [Casey and Gilman, 1988; Neer and Clapham, 1988; Spiegel, 1987; Stryer and Bourne, 1986] by inducing the dissociation of the aβγ heterotrimer and inhibiting the ability of PTX to ADP-ribosylate the α-subunit of G_i. It could be that a defect in this process occurs with diabetes, and the alteration observed with insulin is a reflection of a generalized inability of G_i to be activated. However, different concentrations of Mg^{2+} and GTPγS inhibit PTX-catalyzed ADP-ribosylation to the same degree in liver plasma membranes from diabetic and nondiabetic patients (Fig. 9). It is possible, therefore, that the altered "cross-talk" between the insulin receptor and G_i resides at the insulin receptor level.

We [Caro et al., 1986] and others [Arner et al., 1983] have demonstrated that ^{125}I insulin binding is decreased in liver plasma membranes from obese non diabetic patients, but no difference occurs in ^{125}I insulin binding between obese patients with or without type II diabetes [Caro et al., 1986]. In our patients specific ^{125}I insulin binding (^{125}I insulin 1×10^{-10} M in the presence and absence of unlabelled insulin 1×10^{-6} M) was 0.21 ± 0.05, 0.10 ± 0.02 and 0.12 ± 0.03 ng/mg protein for the lean, obese, and diabetic patients, respectively. We have shown that the major difference between the insulin receptor of obese patients with type II diabetes and lean and obese nondiabetic patients is in the inability of insulin to stimulate the insulin receptor tyrosine kinase activity [Caro et al., 1986, 1987b]. This knowledge is particularly relevant because it has been shown that the purified insulin receptor tyrosine kinase can phosphorylate G_i in cell free systems [O'Brien et al., 1987; Zick et al., 1986]. Rothenberg and Kahn [1988] and Pyne et al. [1989], however, have recently shown that G_i-protein is not a substrate of the insulin receptor tyrosine kinase. However, these studies [Pyne et al., 1989; Rothenberg and Kahn, 1988] do not exclude the possibility that activation of insulin

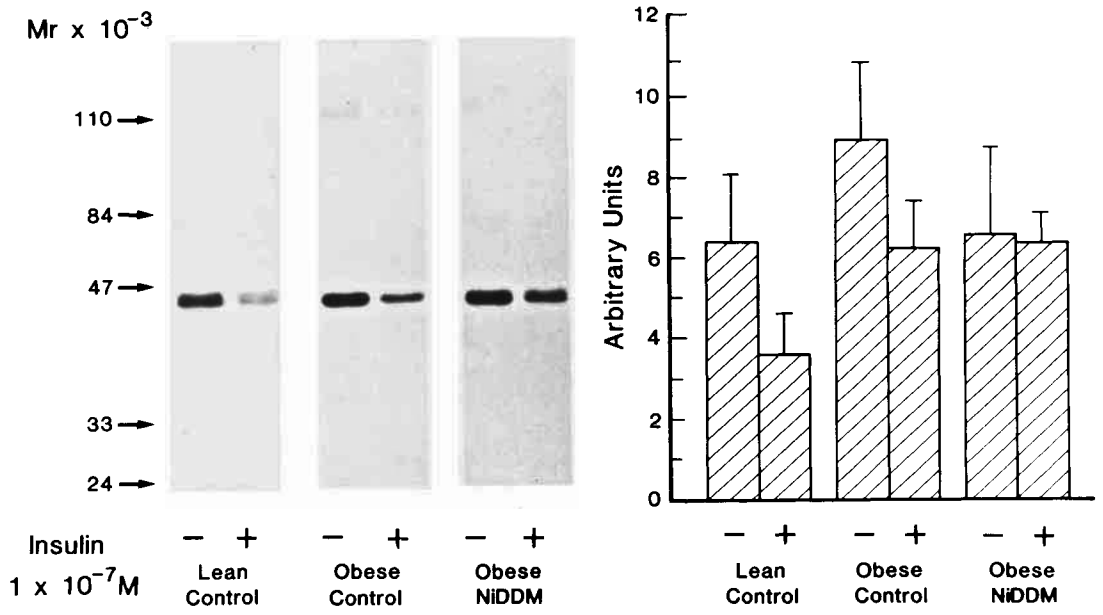

Fig. 8. Effect of insulin on pertussis toxin catalyzed ADP-ribosylation of G_i in lean, obese, and diabetic patients. Experiments were conducted as described in legend of Figure 1. **Left:** Representative autoradiograph of pertussis catalyzed ADP-ribosylation of G_i in the presence and absence of insulin in lean controls, obese controls, and obese diabetic patients. **Right:** Mean ± SEM of the data quantitated by gel densitometer from six different patients in each group.

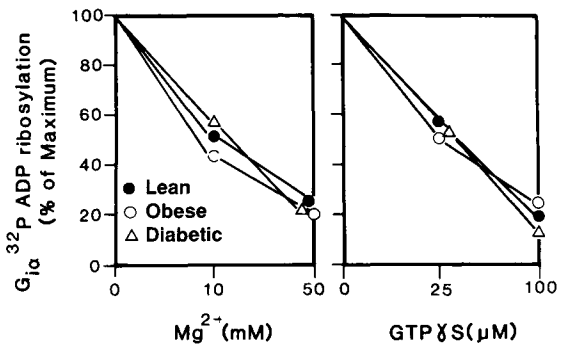

Fig. 9. Effect of Mg^{2+} and GTPγS on pertussis toxin catalyzed ADP-ribosylation of G_i. Pertussis catalyzed ADP-ribosylation was performed as described in Methods in human liver plasma membrane from lean and obese nondiabetic patient and obese diabetic patients in the presence of increasing concentrations of Mg^{2+} (**left**) and GTPγS (**right**). The data represent the mean of two separate experiments.

receptor tyrosine kinase induces conformational changes of the β-subunit of the insulin receptor, which might be a necessary step for the coupling between the insulin receptor and G_i. If true, the alterations of tyrosine kinase in type II diabetes could be the cause of the coupling defect between the insulin receptor and G_i-proteins. This possibility is unlikely, however, because, as shown in Figure 10, an insulin receptor antibody (MA-20) that stimulates insulin action at equimolar concentrations with insulin [14] does not stimulate insulin receptor β-subunit autophosphorylation (middle panel) or insulin receptor tyrosine kinase activity (lower panel) but inhibits PTX-catalyzed ADP-ribosylation of G_i to the same degree that insulin does in human liver (upper panel).

DISCUSSION

One of our research goals is to understand the mechanism(s) of insulin resistance in the liver of obese patients with and without type II diabetes. This study was undertaken because accumulated evidence suggests that G-proteins might be involved in the pathway of insulin action. This evidence can be summarized as follows: (1) PTX, which ADP-ribosylated and inactivates G_i, renders rat adipocytes [Elks et al., 1983; Goren et al., 1985], hepatocytes [Heyworth et al., 1986], BC3H-1 myocytes [Luttrell et al., 1988; Moises et al., 1990], and 3T3-L1 adipocytes [Burdett et al., 1990] resistant to certain responses to insulin; (2) PTX inhibits the insulin-stimulated generation of [^{3}H]myristate-labeled diacylglycerol and hydrolysis of a membrane phosphatidylinositol glycan [Luttrell et al., 1988], which have been proposed to mediate some of insulin's biological effects [Saltiel and Cuatrecasas, 1986]; (3) Burdett et al. [Burdett et al., 1990] have

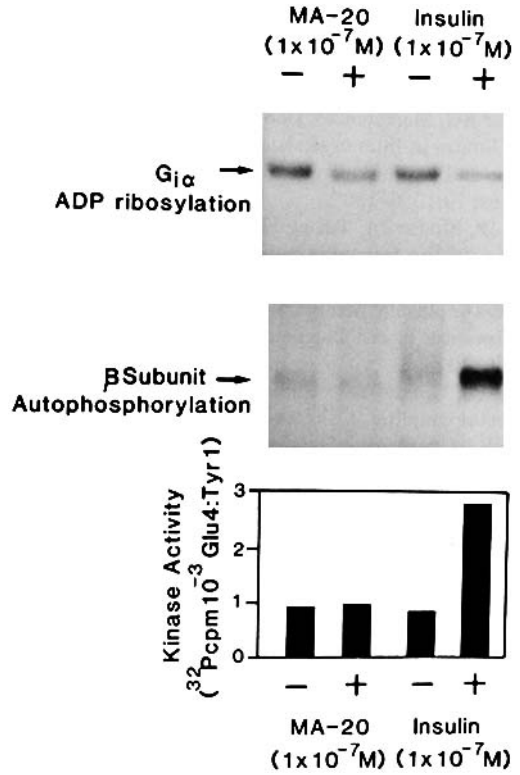

Fig. 10. Effect of insulin and monoclonal antibodies to the insulin receptor (MA-20) on pertussis toxin-catalyzed ADP-ribosylation of G_i (**upper**) insulin receptor β-subunit autophosphorylation (**middle**) and tyrosine kinase activity (**lower**). Experiments were performed as described in Methods. Data in the upper panel were reproduced in three different experiments. The middle panel is from a single experiment and the lower panel is the mean of two separate experiments.

demonstrated G-protein-mediated regulation of the insulin receptor kinase activity in permeabilized LC muscle cells; (4) O'Brien et al. [O'Brien et al., 1989] have shown that insulin increases the phosphorylation on tyrosine residues of a 60-kDa protein that binds to GDP-agarose; (5) Schurmann et al. [Schurmann, 1989] have shown that GTP can inhibit the reconstitution of glucose transport activity in membranes from insulin treated adipocytes; and (6) Davis and McDonald [Davis and MacDonald, 1990] have shown, by using partially purified insulin receptor and isolated adipocytes plasma membranes, that GTP inhibits insulin receptor function but does so by a mechanism not requiring a conventional GTP-binding protein. Finally, the most direct evidence for a functional "cross-talk" between the insulin receptor and G_i is the demonstration that insulin inhibits PTX-catalyzed ADP-ribosylation of G_i in rat liver plasma membrane preparations [Rothenberg and Kahn, 1988].

The studies reported here demonstrate that this novel pathway of functional "cross-talk" between the insulin receptor and G_i is altered in the liver of patients with type II diabetes. As previously demonstrated in rat liver plasma membranes [Rothenberg and Kahn, 1988], insulin inhibits PTX-catalyzed ADP-ribosylation of G_i in liver plasma membranes from nondiabetic patients, but not in liver plasma membranes from diabetic patients. The abnormality in the "cross-talk" between the insulin receptor and G_i-proteins demonstrated in type II diabetes could be due to defect(s) in the insulin receptor, G_i-proteins, or in other factors possibly involved in this process. While the defect of insulin-stimulated receptor kinase activation present in type II diabetes [Caro et al., 1986, 1987a, b; Comi et al., 1987; Freidenberg et al., 1987; Obermajer-Kusser, 1989; Sinha et al., 1987; Takayama, 1988] is a likely candidate, it has been shown in isolated rat hepatocytes that G_i-protein is not a substrate of the insulin receptor tyrosine kinase [Lesko et al., 1973; Rothenberg and Kahn, 1988] and that a monoclonal antibody to the insulin receptor (MA-20) inhibits PTX-catalyzed ADP-ribosylation of G_i without activating insulin receptor tyrosine kinase. Using a system in which the insulin receptor is overexpressed and using a very sensitive assay, Steele-Perkins and Roth [Steele-Perkins and Roth, 1990], were able to demonstrate activation of tyrosine kinase by MA-20. We have been unable to demonstrate stimulation of the insulin receptor kinase activity by MA-20 in the native receptor from human adipocytes [Forsayeth et al., 1987] and hepatocytes, under conditions identical to those in which insulin stimulates insulin receptor tyrosine kinase activity. The study published by Luttrell et al. [1990] clearly demonstrates that the insulin receptor tyrosine kinase has no role in the communication between the insulin receptor and G-protein system, and provides substantial new evidence for a direct noncovalent interaction between the insulin receptor and the regulatory G-protein system.

The second goal of our study was to attempt to estimate the amount of G_i-protein and G_s-protein in liver of obese patients with and without type II diabetes. With regard to G_s-protein, our data from the study of human liver demonstrate an increase in G_s in diabetic patients similar to that noted in animal models of diabetes [Lynch et al., 1989]. However, the available data on G_i-proteins in liver of animal models of

diabetes compared to human liver is more complex. Either >90% reduction in G_i [Gawler et al., 1987] or no reduction [Lynch et al., 1989] has been found in streptozotocin-induced diabetes in rats. No decrease in G_i-protein was found in human liver from patients with type II diabetes compared to lean nondiabetic patients. When diabetic patients are compared with weight-matched controls, a modest but statistically significant decrease in $G_{i1\text{-}2\alpha}$ but not in $G_{i3\alpha}$ is found. Our data suggest that obesity itself is associated with a 2.5-fold increase in $G_{i1\text{-}2\alpha}$ and a 2-fold increase in the $G_{i3\alpha}$. Green and Johnson [Green and Johnson, 1989] have demonstrated a 3-fold increase in G_i in adipose tissue of obese Sprague-Dawley rats. The biological significance of increased G_i-protein in obesity remains to be explained.

We conclude that the novel pathway of insulin signaling involving the regulatory G_i-proteins via biochemical mechanisms not directly involving the tyrosine kinase of the insulin receptor is altered in type II diabetes and offers a new target for the search of the mechanism(s) of insulin resistance.

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