Elevated Plasma Cell Membrane Glycoprotein Levels and Diminished Insulin Receptor Autophosphorylation in Obese, Insulin-Resistant Rhesus Monkeys

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In obese humans, insulin resistance is accompanied by elevated levels of plasma cell membrane glycoprotein (PC-1) and decreased insulin receptor (IR) tyrosine kinase activity in skeletal muscle. PC-1 overexpression inhibits IR tyrosine kinase and possibly other downstream signaling events. The rhesus monkey in captivity is susceptible to obesity with concomitant insulin resistance. In the present study we analyzed obese (n = 10, 29.4% ± 1.2% body fat) and non-obese (n = 12, 19.4% ± 1.9% body fat) rhesus monkeys. Glucose clearance during an euglycemic hyperinsulinemic (400 mU/m^2 body surface area/min) clamp was lower for the obese group (non-obese, 9.7 ± 0.9; obese, 3.2 ± 0.7 mg/kg fat-free mass [FFM]/min; $P < .01$). We performed vastus lateralis muscle biopsies prior to and during the clamp. We measured PC-1 levels in these muscle samples to determine whether PC-1 content is elevated in this primate model of insulin resistance. PC-1 levels were determined by assay of phosphodiesterase activity and specific PC-1 enzyme-linked immunosorbent assay (ELISA). In the obese group, both PC-1 content and activity were 2-fold higher than in the non-obese group ($P < .05$). In order to investigate the ability of insulin to stimulate IR signaling in vivo in these 2 groups of monkeys, we then measured tyrosine autophosphorylation of the IR by specific ELISA. The increase in IR autophosphorylation in the non-obese group was twice that of the obese group (fold increase over basal: non-obese, 3.7 ± 0.3; obese, 1.9 ± 0.6; $P < .05$). We conclude that insulin resistance secondary to obesity in rhesus monkeys is associated with increased levels of PC-1 and decreased IR signaling capacity in skeletal muscle.

RESISTANCE TO the actions of insulin is a common pathological condition in humans and is a major feature of obesity.[1-3] Skeletal muscle is the principal site for insulin-mediated glucose uptake, and insulin resistance in this tissue is responsible for the impaired glucose disposal of obese subjects.[4] Insulin resistance in skeletal muscle has been demonstrated both in vivo and in vitro,[5-8] but the underlying causes remain unknown.

Cellular insulin action is mediated through the insulin receptor (IR), a heterotetrameric, cell surface glycoprotein.[9,10] After insulin binding to the extracellular alpha-subunits of the IR, autophosphorylation of specific tyrosine residues occurs on the transmembrane beta-subunits. This autophosphorylation then activates the protein tyrosine kinase activity of the IR, which phosphorylates key intracellular substrates and initiates the cellular mechanisms involved in enhancing glucose uptake and other responses.[9,11] Most studies of skeletal muscle IR tyrosine kinase activity indicate that this function is impaired in the obese, insulin-resistant state.[12-16] It is likely that in muscle and other tissues of insulin resistant subjects multiple factors contribute to insulin resistance and decreased IR function. Plasma cell membrane glycoprotein (PC-1) and protein tyrosine phosphatase 1B (PTP-1B) have both been demonstrated to reduce IR autophosphorylation.[17-19]

PC-1 is a class II transmembrane protein that is present in most cells and has phosphodiesterase and pyrophosphatase activity.[20] PC-1 is elevated in muscle of obese humans, and elevation of PC-1 in cultured cells inhibits IR tyrosine kinase activation.[21,22] Thus, PC-1 may play a role in certain forms of insulin resistance.

Although there is evidence that PC-1 may play a role in insulin resistance in humans, information on the relationship between PC-1 and IR function in animal models of insulin resistance is scarce. Sakoda et al reported that PC-1 content is elevated in the adipose tissue of the Zucker fatty rat[23] and we have found similar elevations in fat and muscle of the Wistar fatty rat (Youngren et al, unpublished results). However, these elevations of PC-1 do not fully explain the massive insulin resistance in these 2 animal models.

Protein tyrosine phosphatases (PTP) are hypothesized to regulate IR autophosphorylation.[24,25] Adipose and skeletal muscle tissue from obese humans is reported to have higher PTP activity.[26,27] Mice deficient in PTP-1B have increased sensitivity to insulin and decreased adiposity.[18,28] PTP-1B is therefore pertinent to investigations of obesity and insulin resistance.

The rhesus monkey (*Macaca mulatta*) is prone to obesity with subsequent insulin resistance.[29] The obese rhesus monkey has features that resemble human obesity and provides opportunities for longitudinal whole-body measurements, controlled treatment, and mechanistic studies. Longitudinal studies have established that with obesity the monkeys become insulin-resistant with normal plasma glucose levels maintained by elevated plasma insulin levels. Calorie restriction prevents obesity and the development of insulin resistance.[30,31] Thus, the obese rhesus monkey is an important animal model for evaluating the effect of obesity on PC-1 content and IR tyrosine kinase activity in muscle. This study reports elevated PC-1

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content and decreased IR autophosphorylation in obese rhesus monkeys.

METHODS

Animals

We examined 2 groups of rhesus monkeys. The first group consisted of insulin-resistant, obese animals. These animals had been allowed food ad libitum and had an average age of 18 ± 1.8 years (n = 10). The second group was composed of insulin-sensitive non-obese animals. These were either young ad libitum–fed animals of 6.3 ± 0.8 years of age (n = 6), or older chronically calorie-restricted animals of 20.3 ± 0.9 years of age (n = 6) whose caloric intake was restricted such that their weight remained in the same range as the younger animals (≈11 kg).[32] We have previously reported that glucose disposal values in the calorie-restricted monkeys are not significantly different from young ad libitum–fed insulin-sensitive monkeys.[33] In agreement with these findings, statistical analysis confirmed that the younger animals and the calorie-restricted older animals in the present studies were not different with respect to body weight, fasting insulin levels, or insulin-stimulated glucose disposal (data not shown). Thus, both sets of monkeys were combined into the non-obese group. Clinical characteristics including whole-body insulin-mediated glucose disposal rates of monkeys from both groups have been published previously.[31,33,34]

In Vivo Characterization Procedures

All procedures were performed after a 16-hour overnight fast and under light anesthesia (ketamine hydrochloride, 10 mg/kg) unless otherwise noted. Four basal fasting blood samples were collected 3 minutes apart and pooled in order to average previously described rapid oscillations in plasma glucose and insulin levels. Plasma glucose concentrations were determined using the Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Plasma insulin concentrations were measured by radioimmunoassay.[35] Body fat was determined by the tritiated water dilution method.[36,37]

Metabolic glucose clearance was determined by euglycemic hyperinsulinemic clamp as previously described.[38] A priming infusion of regular purified pork insulin was administered, followed by a continuous insulin infusion (>400 mU/m^2 body surface area/min) for 120 minutes to produce maximal insulin-stimulated glucose disposal. Blood samples (0.3 mL) were taken every 5 minutes and a 20% glucose infusion was adjusted to maintain a steady-state plasma glucose level. The animals were initially sedated with ketamine hydrochloride. Anesthesia was maintained by fentanyl citrate (0.01 mg/kg). Succinylcholine (1 mg/kg) was used initially followed by diazepam (2.5 mg/dose) and vecuronium bromide (0.1 mg/kg).

Biopsy Procedure

Vastus lateralis biopsies were performed at 0 and 100 minutes during the euglycemic hyperinsulinemic clamp.[39] Samples were frozen in aluminum tongs cooled in liquid nitrogen within 3 seconds of excision.

Tissue Preparation

Soluble extracts were prepared from frozen muscle tissue. Approximately 500 mg frozen tissue was pulverized at liquid nitrogen temperatures. The resultant powder was homogenized in 2 mL buffer (50 mmol/L HEPES, pH 7.6, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 2 μmol/L leupeptin, 2 μmol/L pepstatin A, 2 mmol/L sodium orthovanadate) at 4°C using a polytron homogenizer (Kinematica, Lucerne, Switzerland) for 8 seconds at a setting of 7. Triton X-100 was added to a final concentration of 1% and the homogenates were solubilized for 60 minutes at 4°C. The material was then centrifuged at 100,000*g* for 60 minutes. The supernatants were collected and stored at –70°C.

PC-1 Activity

The phosphodiesterase activity of PC-1 in solubilized extract from muscle biopsies was measured by the hydrolysis of thymidine 5' monophosphate *p*-nitrophenyl ester (PNTP). The reaction was carried out in 96-well microtiter plates using excess substrate. 20 μg protein from each sample was incubated with 500 nmol PNTP in 125 μL buffer (0.1 mol/L 2-amino-2-methyl-propanol, 7.5 mmol/L magnesium acetate, pH 9.4) at 37°C for 90 minutes. Care was taken to ensure that the same amount of tissue homogenization buffer was present for each sample. Liberated *p*-nitrophenol was quantified by optical absorption measurements at 401 nm by a microtiter plate reader.

PC-1 Content

Protein content of solubilized samples was determined by the bicinchoninic acid (BCA) method with bovine serum albumin (BSA) as the standard (Pierce, Rockford, IL). PC-1 in solubilized muscle extract was determined by specific enzyme-linked immunosorbent assay (ELISA) as described previously.[40] Briefly, 96-well immuno Maxisorp plates (Nunc, Denmark) were coated with 2 μg/mL anti–PC-1 antibody in 20 mmol/L sodium bicarbonate, pH 9.6. After washing 3 times with Tris-buffered saline with Tween (TBST) (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20) the wells were blocked with 2% BSA in TBST at 56°C for 30 minutes. The plate was washed again 3 times with TBST and 20 μg protein from solubilized muscle extract in binding buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Triton X-100, 0.1% BSA, 1 mmol/L PMSF, 2 μmol/l pepstatin A, 2 μmol/L leupeptin) was allowed to bind for 18 hours at 4°. After being washed 5 times with TBST, the plate was incubated with biotin-conjugated monoclonal PC-1 antibody at 1:3,000 dilution in buffer B (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20, 1% BSA, 1 mg/mL bacitracin, 1 mmol/L PMSF) 22°C for 2 hours. The plate was then washed 5 times in TBST followed by 0.1 μg/mL streptavidin-horseradish peroxidase (HRP) diluted in buffer B. The assay sensitivity was enhanced using ELAST reagent (Du Pont-NEN, Boston, MA), and peroxidase quantified by TMB reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD) with absorbency read at 450 nm.

IR Content

IR levels were measured by IR ELISA as previously reported[21] using the same procedure described above with the following modifications. IR from 30 μg protein of solubilized muscle extract was immunocaptured using the monoclonal IR antibody CT-1. IR quantities were reported using biotinylated monoclonal IR antibody 2G7 (kindly provided by R. Roth, Stanford University, CA). The IR signal was enhanced using ELAST reagent.

IR Autophosphorylation

The tyrosine phosphorylation of IR in extracts of muscle biopsies obtained prior to and during insulin infusion was measured by ELISA as described previously[16] using the procedure outlined above. Insulin receptor from 5 to 10 μg protein of solubilized muscle extract was immunocaptured as for the IR ELISA. Tyrosine phosphorylation of immunocaptured IR was measured via biotinylated anti–phosphotyrosine (anti-Py) antibody (Upstate Biotechnology, New York). The signal was enhanced using ELAST reagent.

PTP-1B Content Measurement

PTP-1B content was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 30 μg soluble protein diluted in Laemmli reducing buffer, denatured, and loaded onto an 8% to 16% polyacrylamide mini-gel. The samples were loaded as single determinations and run through the gel at 30 mA for 1 hour. The

proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V for 1.6 hours. Membranes were blocked with 5% nonfat milk in TBST, washed 3 times in TBST, and probed using polyclonal antibody to PTP-1B (Upstate Biotechnology, New York) at 1:1,000 dilution in TBST. After a 2-hour incubation at 22°C or overnight incubation at 4°C, membranes were washed and incubated with anti-rabbit IgG-conjugated HRP (1:3,000 dilution in TBST) for 90 minutes at 22°C. Membranes were then washed, incubated with enhanced chemiluminescent (ECL) detection reagents for 5 minutes, and immediately exposed to x-ray film. The 50-kd band was quantified by optical densitommetry (Alpha Innotech, San Leandro, CA).

Data Analysis

Data are expressed as means ± SE and were analyzed statistically using Student's *t* test. Differences between groups were considered significant at $P < .05$.

RESULTS

Characteristics of the Animals

Twelve non-obese animals and 10 obese animals were studied. There were significant differences in the average weight and percent body fat for the 2 groups (Table 1). In the non-obese animals, the fasting insulin and the fasting glucose levels were approximately half the values measured in the obese group.

The degree of insulin resistance in these animals was measured by euglycemic, hyperinsulinemic clamp. The metabolic clearance of glucose was significantly reduced in the obese animals (3.2 mg/kg fat-free mass [FFM]/min) compared with the non-obese animals (9.7 mg/kg FFM/min) (Fig 1). Impaired muscle insulin signaling was confirmed in the obese group by comparing previously published skeletal muscle glycogen synthase activity.[33,39] Basal and insulin-stimulated glycogen synthase total activity (measured in the presence of 10 mmol/L glucose-6-phosphate [G6P]) was significantly higher in the non-obese monkeys compared to the obese monkeys (basal non-obese *v* obese: 19.1 ± 1.2 *v* 11.6 ± 0.9 nmol/min/mg protein, $P < .0001$; insulin-stimulated non-obese *v* obese: 20.8 ± 1.6 *v* 10.8 ± 0.9 nmol/min/mg protein, $P < .0001$). Basal and insulin-stimulated glycogen synthase–independent activity (measured in the presence of 0.1 mmol/L G6P) was also significantly higher in the non-obese monkeys compared to the obese monkeys (basal non-obese *v* obese: 2.7 ± 0.6 *v* 1.1 ± 0.4 nmol/min/mg protein, $P < .05$; insulin-stimulated non-obese *v* obese: 4.1 ± 0.6 *v* 1.7 ± 0.5 nmol/min/mg protein, $P = .01$).

Table 1. Characteristics of the Animal Groups

	Non-Obese	Obese
N	12	10
Body fat (%)	19.4 ± 1.9*	29.4 ± 1.2
Weight (kg)	10.5 ± 0.5*	15.5 ± 1.1
Fasting plasma insulin (pmol/L)	295 ± 38†	712 ± 175
Fasting plasma glucose (mmol/L)	3.7 ± 0.1†	6.7 ± 1.3

NOTE. Values are the mean ± SEM.
*$P < .01$.
†$P < .05$.

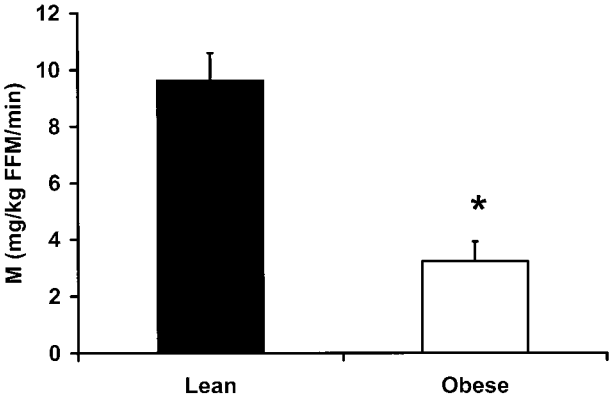

Fig 1. Decreased metabolic glucose clearance (M) in obese rhesus monkeys measured by euglycemic hyperinsulinemic clamp (N = 22). *Value significantly different from non-obese control, $P < .01$.

PC-1 Levels in Non-obese Versus Obese Monkeys

Vastus lateralis biopsies were obtained during the glucose clamp procedure both prior to and at 100 minutes' insulin infusion. Studies were carried out to measure PC-1 levels in the biopsies from the obese and non-obese groups of animals. PC-1 was first measured enzymatically employing the hydrolysis of the substrate thymidine 5' monophosphate p-nitrophenyl ester (PNTP). PC-1 activity was 2-fold greater in the obese versus the non-obese animals (Fig 2A). We also measured PC-1 content by specific ELISA. Muscle PC-1 content was significantly elevated in the obese animals, with both assays producing similar results (Fig 2B). There was no correlation between PC-1 levels and glucose levels in the animals.

IR Content and Function in Non-obese Versus Obese Monkeys

IR content was measured by specific ELISA in the obese and non-obese animals. There was no significant difference in muscle IR content between groups (Fig 3). Next we measured IR signaling by an IR autophosphorylation ELISA in biopsies obtained under basal and insulin-stimulated conditions. The ability of insulin to stimulate IR autophosphorylation was significantly impaired in the obese animals. The insulin-induced increase in autophosphorylation over basal levels was reduced approximately 50% in the obese versus non-obese group (Fig 4).

PTP-1B

We measured PTP-1B content by Western blot in muscle samples obtained under basal conditions from non-obese and obese animals. The PTP-1B content of muscle did not differ between the 2 groups (Fig 5).

DISCUSSION

Earlier studies have reported that rhesus monkeys in captivity become obese when allowed free access to food. Concomitant with obesity these animals develop hyperinsulinemia and overt diabetes.[35] Prior investigations in these animals have shown that distal effector systems, especially glycogen syn-

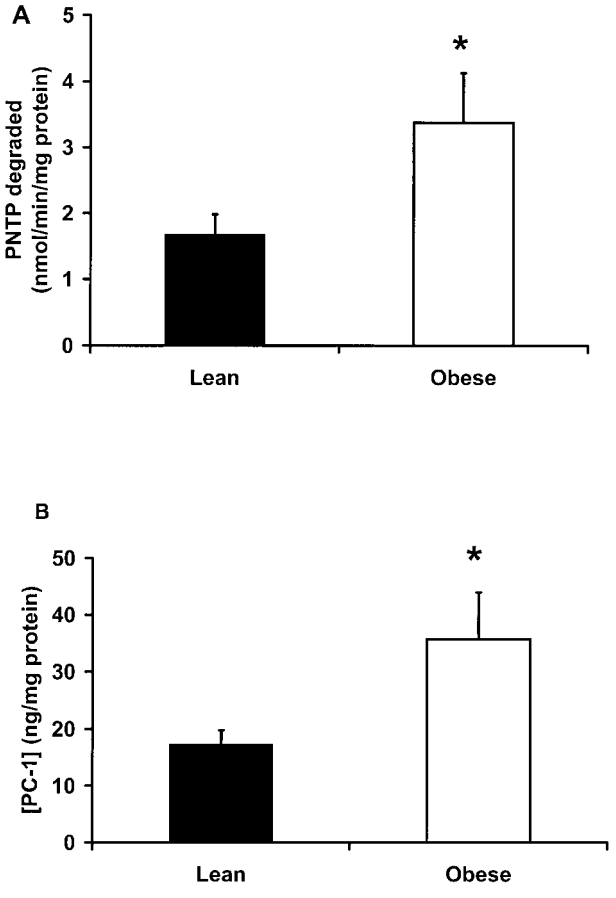

Fig 2. Elevated PC-1 in muscle from obese rhesus monkeys. (A) PC-1 enzymatic activity measured by phosphodiesterase activity. Rates of hydrolysis of the phosphodiesterase substrate, PNTP, were 1.7 ± 0.3 and 3.4 ± 0.8 nmol/min/mg, non-obese and obese, respectively. (B) PC-1 content determined by ELISA. The PC-1 content was 17.3 ± 2.5 and 35.8 ± 8.2 ng/mg in the non-obese and obese groups, respectively. *Values significantly different from non-obese controls, *P* < .05.

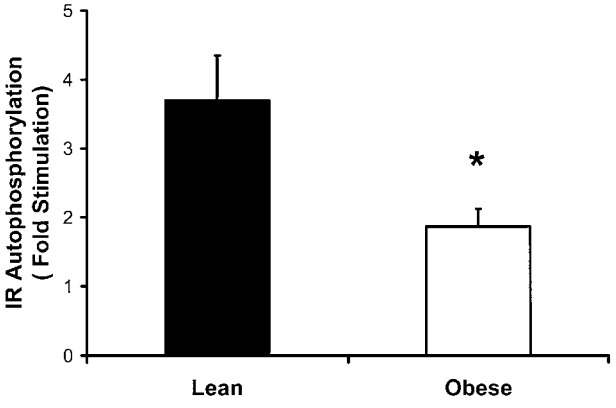

Fig 4. Impaired muscle IR autophosphorylation in obese rhesus monkeys. Tyrosine phosphorylation of IR from muscle biopsies obtained under basal and insulin-stimulated conditions was determined by specific ELISA. Muscle biopsies were obtained prior to and during a maximally stimulating euglycemic, hyperinsulinemic clamp. IR was immunocaptured by monoclonal IR antibody and the amount of phosphotyrosine was reported by biotinylated anti-pY. The average fold increase over basal was 3.7 ± 0.6 and 1.9 ± 0.3 for the non-obese and obese groups, respectively. *Values significantly different from non-obese control, *P* < .05.

thase, demonstrate an impaired response to in vivo insulin stimulation.[39] However, proximal pathways, including the function of the IR itself, have not been analyzed. In the present study we measured insulin sensitivity in non-obese and obese rhesus monkeys by euglycemic hyperinsulinemic clamp, and obtained skeletal muscle biopsies at 0 and 100 minutes into the insulin infusion. Metabolic glucose clearance was markedly decreased in the obese animals. In monkeys, as in humans, muscle is the major tissue for insulin-mediated glucose disposal. Accordingly, we measured the IR content and function in muscle from non-obese and obese animals in order to address potential mechanisms that could contribute to insulin resistance in this tissue. There was no change in IR content between the 2 groups. However, when IR autophosphorylation was mea-

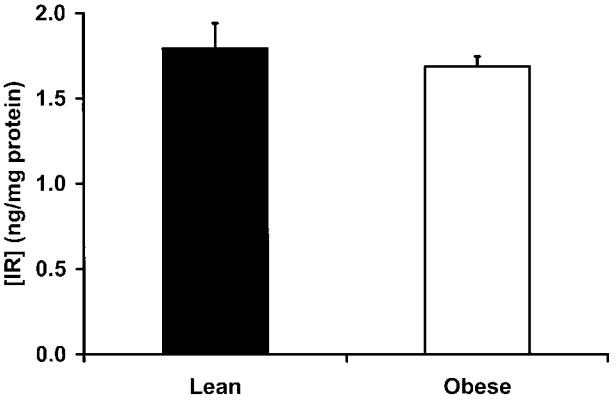

Fig 3. Muscle IR content in rhesus monkeys. IR content in soluble muscle extracts measured by specific ELISA. No significant difference was observed between groups.

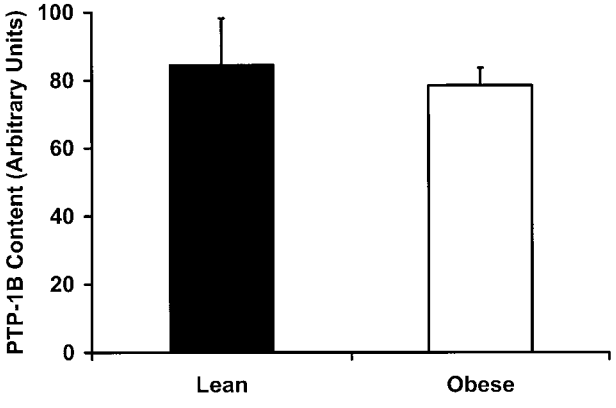

Fig 5. Muscle PTP1-B content in rhesus monkeys. PTP1-B content of soluble muscle samples was determined by Western blot and scanning densitommetry. No significant difference was observed between groups.

sured, the obese animals had an approximate 50% decrease in this function. In many but not all studies of obese humans, IR tyrosine kinase activity is decreased in muscle and fat.[15,16,41] Thus, this parameter in obese monkeys appears to resemble obese humans. While decreased IR function is a common finding in the insulin-resistant state, the mechanisms of this impairment are not well understood. Two potential inhibitors of the IR are PC-1 and PTP-1B.

In the present study, we measured muscle PC-1 content in non-obese and obese rhesus monkeys. We found that in obese animals PC-1 content was increased. PC-1 was measured by both ELISA and assay of phosphodiesterase activity. These results are in agreement with our previous work with obese humans showing a correlation between PC-1 and obesity.[21]

PC-1 is a class II transmembrane glycoprotein that is located both on plasma membrane and in the endoplasmic reticulum. PC-1 exists as a homodimer of 230 to 260 kd, and it is inserted into the membrane such that there is a small cytoplasmic NH_2-terminus and a larger extracellular COOH-terminus.[20] In vitro, PC-1 is overexpressed in fibroblasts from both diabetic and nondiabetic insulin-resistant subjects.[17] In these cells, maximal IR tyrosine kinase activity is concomitantly decreased.[17] In several cultured cell types, when PC-1 is overexpressed by transfection, IR tyrosine kinase activity and subsequent IR signaling are decreased.[17,42,43] These studies suggest, therefore, that PC-1 is a unique cellular inhibitor of IR signaling. In vivo studies of insulin-resistant subjects demonstrate that PC-1 is elevated in fat and muscle.[40,44] The elevation of PC-1 in muscle from obese subjects correlates with decreased insulin stimulation of glucose transport.[21] There was no relationship between PC-1 levels and glucose levels in these patients. This finding was similar to the data obtained with the animals in the present study. PC-1 is also elevated in muscle of obese patients with gestational diabetes and this elevation correlates with decreased IR function.[45]

We recently have shown that PC-1 associates with the IR.[22] Luo et al have recently elucidated the quaternary structure of the IR by scanning transmission electron microscopy.[46] They found that a region on the surface of the IR molecule, termed the connecting domain, functions as a hinge, transmitting the hormone signal from the occupied ligand-binding domain to the catalytic domain. We have found that PC-1 does not interact with an IR deletion mutant in the connecting domain.[22] These data suggest, therefore, that PC-1 interacts at this site on the IR, and thus may prevent the insulin-induced conformational change of the alpha-subunit. Therefore it is possible that elevated PC-1 may contribute to the decreased IR tyrosine kinase found in obese monkeys.

PTP are hypothesized to regulate IR autophosphorylation.[24,25,47] Skeletal muscle tissue from obese humans are reported to have higher PTP activity.[27] Mice deficient in PTP-1B have increased sensitivity to insulin and decreased adiposity.[18,28] We measured PTP-1B content by Western blot and found no difference between obese and non-obese animals. These data in rhesus monkeys are in contrast to findings in humans and suggest that in rhesus monkeys insulin resistance accompanying obesity is not associated with increased PTP-1B levels. Insufficient amounts of samples prevented the measurement of PTP-1B enzyme activity. It is possible that PTP-1B activity was elevated in these muscle tissues, and further studies will be needed to clarify this point.

The reduction in the metabolic clearance of glucose in the obese monkeys was much more dramatic than the 50% decrease in IR autophosphorylation observed in this group. While the stoichiometric relationship between IR signaling and the biological effects of insulin are unknown, this observation suggests that factors other than IR tyrosine kinase may operate in the obese monkey to induce insulin resistance. One possibility is that PC-1 has other effects on the IR signaling system in addition to inhibiting the IR itself. In one study with cultured cells, the data suggested that PC-1 overexpression could inhibit insulin stimulation of glucose transport and S6 kinase activity independently of PC-1's effects on IR function.[42] Another likely possibility is that other factors are operative in obesity and inhibit the IR signaling pathway or the glucose transport effector system in muscle. For example, the non-obese monkeys in the present study had approximately 2-fold higher total and independent activity of glycogen synthase compared to the obese monkeys. In a previous study, we showed that total and independent activity of skeletal muscle glycogen synthase were related to whole-body insulin-mediated glucose disposal rates in monkeys varying in degree of insulin sensitivity.[39] Therefore, both an increase in PC-1 content and a decrease in glycogen synthase activity in the obese monkeys may be contributing to whole-body insulin resistance.

Prior studies of the obese insulin-resistant rhesus monkey have provided important information concerning skeletal muscle insulin resistance and the effect of this impairment of function on the metabolism of glucose and other substrates.[48] The present study demonstrates alterations in PC-1 in obese rhesus monkeys. These animals therefore may also provide important information on the role of PC-1 in insulin resistance.

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